Effects of sialic acids in immunity

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Above: Wassily Kandinsky; *Accent in pink* (1926).

Chapter 1

General Introduction

The Immune system

The immune system is a complex network of cells, tissue and organs designed to protect the body from a variety of invading pathogens including bacteria, viruses, fungi and parasites. Additionally, the immune system recognizes and destroys host's cells that are dysfunctional such as transformed cells growing abnormally, representing thus a mechanism of defence against the development of tumors. At the same time, the immune system provides immunological tolerance towards self as well as innocuous foreign antigens by avoiding excessive and unwarranted immune reactions.

The immune system consists of two main parts: the innate immune system and the acquired immune system. These two branches co-operate in order to detect and eradicate the danger. The innate immune system, also known as non-specific immunity, represents the first line of defence providing a rapid and none antigen-specific response against pathogens. This arm of the immune system comprises natural killer (NK) cells, dendritic cells (DCs), macrophages and granulocytes as well as a humoral part represented by the complement system.

If pathogens successfully escape from the innate immune cells, a new barrier of protection is represented by the acquired or adaptive immune system. In contrast to the innate immune response, the acquired arm of the immune system responds in an antigen-specific manner and builds up protection through immunological memory, which enables the infected host to mount a faster and a stronger response each time the same pathogen is encountered[1]. The acquired immune system comprises highly specialized cells such as B cells and T cells. B cells play an important role in humoral immune responses to unwanted invaders. Recognition of harmful antigens by B cells induces their activation and differentiation into plasma cells that secrete antigen-specific antibodies, which neutralize or tag microbes and infected cells for attack by other cells of the immune system such as macrophages and neutrophils. T cells are major components of the acquired immune system. In contrast to B cells, which recognize their cognate antigen in its native form, T cells recognize antigens as peptide fragments that are processed and presented by antigen presenting cells (APCs) such as DCs. The T cell compartment can be subdivided in $CD4^+$ T cells and $CD8^+$ T cells.

Following activation by APCs, naive $CD4^+$ T cells differentiate into distinct antigen-specific T helper sub-lineages with unique features to 'help' other components of the immune system such as $CD8^+$ T cells, macrophages and B cells[2,3]. Upon activation, naive $CD8^+$ T cells become cytotoxic T lymphocytes with the ability to kill virally infected or dysfunctional cells[4].

Dendritic cells and initiation of T cell response

DCs play a crucial role in the initiation of the acquired immune response. DCs are defined as professional APCs because they are capable of processing and presenting antigens to naive T cells in order to tailor the acquired immune response to match the antigenic setting[5-7]. More specifically, following uptake antigens are processed by the proteasome or in lysosomes into small peptides that are loaded onto major-histocompatibility complexes (MHCs). Peptide- loaded MHC molecules are exposed on the DC surface allowing the recognition by T cell receptor (TCR) present on naive antigen-specific T cells. MHCs include MHC class I (MHC-I) molecules, which present peptides from endogenous proteins to $CD8^+$ T cells, and MHCclass II (MHC-II) molecules, which are loaded with extracellular protein-derived peptides for the interaction with $CD4^+$ T cells[8]. In addition, DCs have the specialized capacity to present exogenous antigens in the context of MHC-I for the activation of $CD8^+$ T cells in a process known as cross-presentation[9]. This process is necessary to initiate $CD8^+$ T cell-mediated immune response against tumors and viruses that do not infect DCs[10].

DCs originate from precursors in the bone marrow and migrate at various differentiation stages to peripheral tissues, e.g. skin, respiratory and gastrointestinal tract, where they reside in an immature state and continuously survey the environment[11]. In the steady state, immature DCs induce and maintain immune tolerance when sampling harmless antigens such as self antigens. However, when encountering a pathogen, DCs undergo maturation that is necessary to initiate a protective T cell-mediated immune response. Maturation of DCs results from changes of the DC phenotype. DC maturation primarily occurs upon perception of pathogens by DCs through the pattern recognition receptors (PRRs). PRRs recognize pathogen- associated molecular patterns (PAMPs) shared by broad

groups of microorganisms[12]. The recognition of PAMPs such as lipopolysaccharide (LPS) and double stranded viral RNA by PRRs induce the up-regulation of co-stimulatory proteins on DCs including CD80 and CD86, which bind counter receptors expressed on T cells[13]. Moreover, mature DCs secrete pro-inflammatory cytokines and other polarizing co-stimulatory molecules that induce then the proper T cell response[14]. Thus, the presentation of pathogen-derived antigen epitopes in the context of MHC molecules together with the DC maturation induced upon triggering PRRs represent the essential signals for the initiation of an effector T cell response against pathogens.

Cytotoxic $CD8^+$ T cells

$CD8^+$ T lymphocytes are important mediators of adaptive immunity against intracellular pathogens such as viruses, bacteria and parasites as well as tumor cells. In case of a viral infection or during tumorigenesis, mature DCs migrate to secondary lymph nodes to present antigens in the context of MHC-I for the interaction with naive $CD8^+$ T cells[15]. The TCR/MHC-I interaction together with co-stimulatory signals given via CD40 and CD70 induce the differentiation of naive $CD8^+$ T cells into Cytotoxic T Lymphocytes (CTLs), which are licensed to kill virally infected or tumor cells by establishing an interaction with peptide- loaded MHC-I molecules expressed on the target cell[16] (Fig. 1 A). Following interaction with a target cell, CTLs release cytotoxins such as perforin and Granzyme B (GrB), which eventually lead to apoptosis[17,18]. CTLs also secrete effector cytokines such as IFN-γ and TNF-α, which directly inhibit viral replication and potently stimulate the activity of macrophages to mediate clearance of ingested pathogens and/or cellular debris and accordingly resolve the infection[19].

$CD4^+$ T helper

The recognition of peptide-loaded MHC-II molecules by naive antigen-specific $CD4^+$ T cells together with co-stimulatory and polarizing signals provided by DCs promotes expansion and differentiation of $CD4^+$ T cells into T helper (Th) cells[20]. According to the type of pathogen encountered, DCs undergo a differential maturation program that properly generates a highly specialized Th subset with

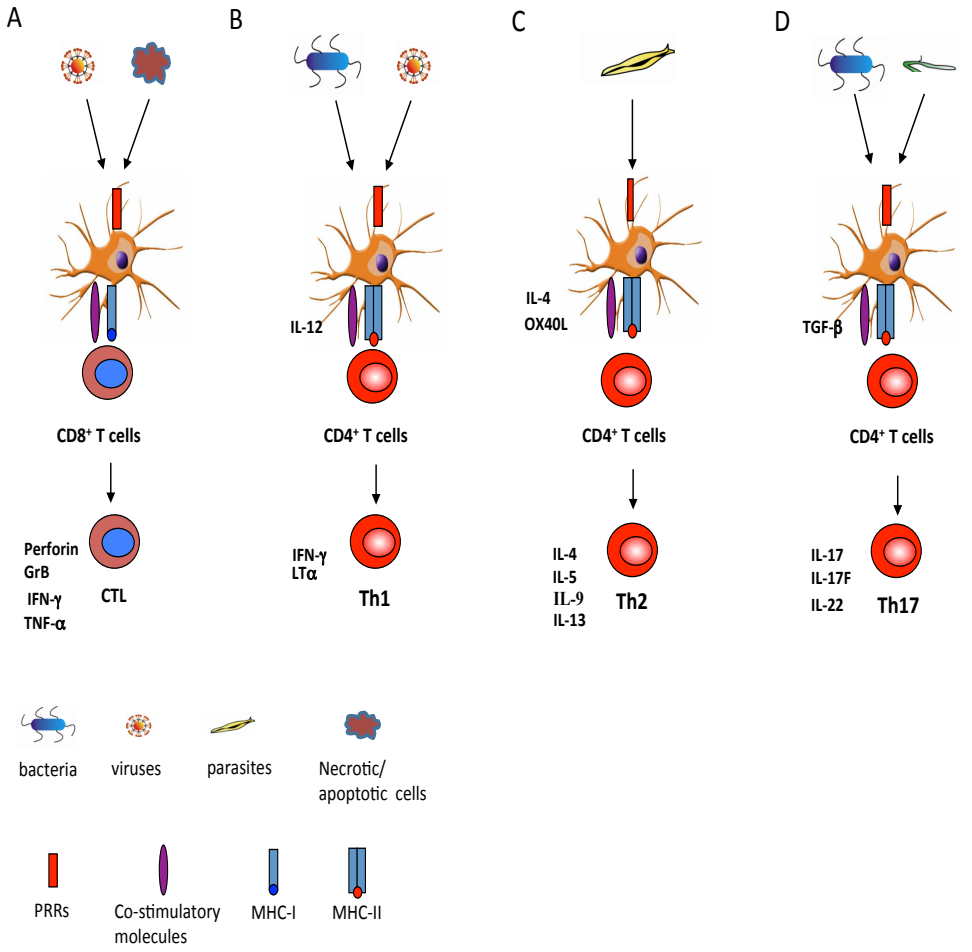

Figure 1: T cell differentiation by DCs. DCs express a repertoire of antigen uptake receptors and TLRs that are both involved in the recognition of pathogen and tumor antigens. (A) During a viral infection or tumor formation, the presentation of intracellular antigens in the context of MHC-I together with up-regulation of co-stimulatory molecules induces naïve $CD8^+$ T cells to differentiate into CTLs that are able to kill virally infected or transformed cells through the secretion of perforin, GrB, and cytokines such as IFN-γ and TNF-α. (B-D) If antigen is loaded into MHC-II with the simultaneous expression of co-stimulatory molecules by DCs, naive $CD4^+$ T cells differentiate into Th. The uptake of bacteria or viruses by DCs induced generation of Th1 (B), parasites promote generation of Th2, bacteria and fungi lead to Th17 generation (C).

distinct and unique features. In particular, intracellular pathogens including bacteria, viruses, parasites and yeasts, drive DCs to produce interleukin (IL)-12, which, under influence of the transcription factor T-bet, favors the differentiation of naive $CD4^+$ T cells into the Th1 lineage (Fig. 1 B). Th1 cells exert microbicidal activity and stimulate functions of CTLs, NK cells and macrophages by secreting IFN-γ and lymphotoxin LTα[21]. Secretion of IL-4 and expression of OX40L by DCs encountering helminthes or other extracellular parasites promote expression of the transcription factor GATA-3 into naive $CD4^+$ T cells leading to Th2 commitment[22,23] (Fig. 1 C). Differentiated Th2 cells produce IL- 4, IL-5, IL-13 and IL-9 that have effects on many cell types in the body including mast cells, eosinophils and basophils. In addition, Th2 also induce B cell-class switching to IgE, which stimulates mast cells to release histamine, serotonin, and leukotrienes causing a local inflammatory response and recruiting basophils to contribute in the defense against these pathogens. Over-activation of Th2 cells is associated with allergic inflammatory diseases including asthma, allergic rhinitis and atopic dermatitis[24].

Besides Th1 and Th2 cells, $CD4^+$ T cells can also polarize into Th17 cells, which secrete the pro-inflammatory cytokines IL-17, IL-17F and IL-22[25] (Fig. 1 D). Th17 cells play an important role in the host's defense against microbial and fungal infections by recruiting neutrophils and macrophages to the infected tissues. However, Th17 cells have been implicated in the pathogenesis of neuroautoimmune diseases, especially in multiple sclerosis (MS) and the Guillain-Barr syndrome (GBS) as well as in their animal models, experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN)[26,27].

Studies in vitro in mice and revealed that the combination of transforming growth factor-β (TGF-β) and cytokine IL-6 are required for Th17 differentiation via commitment of the transcription factor RoRγT[28], while the cytokines IL-23, IL-1β and TNF-α are important for amplifying and/or stabilizing Th17 phenotype[29,30]. In humans, the signaling leading to Th17 differentiation is still unclear, although an involvement of IL-21 and TGF-β has been shown[31]. Other Th subsets that have recently been identified and include T follicular helper (Tfh)[32], Th9[33], and Th22[34]. In conclusion, differentiation of naive $CD4^+$ T cells to

distinct functional Th lineages permits the generation of a unique and specialized immune response against pathogens, while minimizing tissue damage.

Regulatory T cells: biology, functions and implications in autoimmunity

The absence or reduced expression of co-stimulatory molecules as well as expression of inhibitory molecules and cytokines by DCs upon uptake of self or harmless foreign antigens induce naive T cells to differentiate into regulatory or suppressive T cells (Tregs). Tregs play a pivotal role in maintaining immune homeostasis by avoiding/preventing unwarranted and excessive reactions that would be harmful for the host, affording thus immune tolerance. Different types of Tregs have been identified (Table I). One subset develops during the normal process of T cell maturation in the thymus, resulting in a population of antigen- specific Tregs known as naturally occurring Tregs (nTregs). The concept of a thymus-derived T cell population with suppressive abilities began in the early 1970s[35,36], but only many years later nTregs were phenotypically defined as a $CD4^+$ T cell subset highly expressing CD25 (the IL-2 receptor a-chain) and characterized by the expression of the transcription factor Forkhead box P3 (FoxP3), which is essential for their development and function[37-39]. In addition, other markers have been associated with nTregs; these include the cytotoxic T- lymphocyte antigen-4 (CTLA-4)[40], the glucocorticoid-induced TNFR family related gene (GITR)[41], the lymphocyte activation gene-3 (LAG-3)[42] and the ectonucleotidases CD39 and CD37[43].

nTregs mainly exert their immunosuppressive functions in a cell-contact dependent manner. The assumption of a cell-contactgt-mediated suppression by nTregs is derived from *in vitro* experiments showing that nTregs could not suppress effector T cell function when separated via a semi-permeable membrane[44]. One of this cell-contact mechanisms of immunosuppression is represented by the constitutive expression of CTLA-4 on nTreg[45].

CTLA-4 on nTreg binds the co-stimulatory molecules CD80 and CD86 and outcompetes the activating receptor CD28 on naive and/or effector T cells for binding these co-stimulatory molecules, thus abrogating the activating signal

for effector T cells. Another cell-contact dependent mechanism described involves LAG-3, which binds MHC-II on DCs triggering an ITAM-mediated signaling pathway in DCs that impairs DC pro-inflammatory functions[46].
However, another immunosuppressive function of Tregs is now emerging. This includes the abrogation of extracellular ATP by CD39, which is an ctonucleotidase that hydrolyzes adenosine-5'-triphosphate (ATP) to adenosine diphosphate ADP) or adenosine monophosphate (AMP)[47]. In the immune system, ATP functions as danger signal and indicator of tissue damage; upon pathogen infection or

Table I: Subsets of natural and induced $CD4^+$ and $CD8^+$ Tregs detected in humans and rodents.

nTreg

	Transcription factor expressed	Other associated markers	Target cells	Regulatory mechanism	References
$CD4^+$ Treg	FoxP3	CD25hi, CTLA4, GITR,	T cells, APCs	Cell-contact dependent via CTLA4	37-43
$CD8^+$ Treg	(?)	CD44, ICOSL	Tfh	Qa1-restricted suppression	64-69
$CD8^+$ $CD28^-$ Treg	(?)	CD25	Tcells, DCs/APCs	Mediated by IL-10/TGF-β, Induction of ILT3/ILT4 in DCs	70-71

iTreg

	Transcription factor expressed	Other associated markers	Target cells	Regulatory mechanism	References
$CD4^+$ Treg	FoxP3 Helios?	CD25, CTLA4, GITR, CD39,CD37	T cells	Cell-contact dependent via CTLA4, mediated by IL-10 and TGF-β, IL−35	52,58

tumor formation, the damaged cells release high amounts of ATP, which binds purinergic P2 receptors that are highly expressed on immune cells[48]. These receptors induce maturation of DCs and release of IL-1β by macrophages and DCs[49]. Therefore, the degradation of extracellular ATP by CD39 on Tregs inhibits the pro-inflammatory functions of the innate immune cells and their capacity to initiate Th1 response, promoting thus immunosuppression[50]. In addition, it has also been shown that CD39 generates adenosine in concert with the ecto-5'-nucleotidase CD73, which is also constitutively expressed on nTregs[51]. AMP generated by CD39 is in turn degraded by CD37 to adenosine, which binds A2A adenosine receptor on T cells. This interaction promotes the suppression of T cell effector functions[52].

Besides nTregs, naive $CD4^+$ T cells can differentiate in the periphery or in vitro into "adaptive" or "induced" Tregs (iTregs) upon particular conditions of antigen stimulation[53]. Simultaneous exposure of DCs to TLR ligands and anti-inflammatory mediators such as IL- 10, TGF-β or retinoic acid (RA) differentiate DCs into tolerogenic DCs (tDCs). Subsequently, tDCs themselves produce anti-inflammatory mediators or overexpress inhibitory molecules such as Immunoglobulin-like transcript 3/4 (ILT-3/4)[54], Programmed death-ligand1/2 (PD-L1/2)[55] and Indoleamine 2, 3-dioxygenase (IDO)[56], which polarize T cells towards iTregs or induce unresponsiveness. In the intestinal mucosa, the cross-talk between intestinal epithelial cells and DCs leads to tDCs development, which promotes $CD4^+$ $FoxP3^+$ iTregs generation in order to provide tolerance towards commensal bacteria and food antigens[57].

Unlike nTregs, iTregs do not constitutively express FoxP3 or high levels of CD25, yet the expression of these markers is variable depending on the disease setting and the nature of the inflammatory response. The best defined iTregs are $CD25^{hi}$ $FoxP3^+$, Th3 and type 1 regulatory T cells (Tr1). $CD25^{hi}$ $FoxP3^+$ iTregs mainly resemble nTregs both in the expression of surface molecules such as CTLA-4, GITR and CD39 and in the mechanisms of immunosuppression[58]. Th3 cells develop after low dose oral antigen exposure, and they are involved in mucosal immunity and protecting mucosal surfaces in the gut from harmless non- self antigens[59]. Th3 secrete TGF-β, suppress Th1 and Th2 functions in an antigen-non specific manner and have been shown to inhibit

EAE[1]. Tr1 cells were first discovered in a Severe Combined immunodeficiency (SCID) patient transplanted with fetal liver stem cells[60]. Tr1 can be generated in vitro upon antigen stimulation in the presence of IL-10 or by treatment of mice with rapamycin or vitamin D3 plus dexamethasone[61,62]. Tr1 cells do not constitutively express FoxP3, yet produce high quantities of IL-10 and little IFN-γ[63].
Although $CD4^+$ Tregs have garnered much attention for their role in the maintenance of the immune homeostasis, the concept of Tregs has now also been expanded to $CD8^+$ T cells[64]. In mice, the depletion of $CD8^+$ T cells or lack of the CD8 molecule in mice increases the severity of experimental auto-immune diseases such as EAE, auto-immune myocarditis and collagen-induced arthritis[65-67]. These effects could be attributed to a thymus-derived $CD8^+$ T cell subpopulation with immunosuppressive abilities ($CD8^+$ nTregs) has been identified in humans and rodents. These natural $CD8^+$ Tregs express CD44 and the inducible costimulator ligand (ICOSL) molecule and mainly suppress Tfh cell responses in a manner involving the non-classical MHC class 1b (Qa-1)[68]. The genetic disruption of the interaction between the TCR of $CD8^+$ Tregs and Qa-1^+ on Tfh results in the development of a lethal Systemic Lupus Erythematosus (SLE)-like disease[69]. Other defined natural $CD8^+$ Tregs are the $CD8^+$ $CD28^-$ Tregs. Unlike $CD4^+$ nTregs and $CD8^+$ $ICOSL^+$ $CD44^+$ nTregs, $CD8^+$ $CD28^-$ nTregs exert their immunosuppressive functions by secreting soluble mediators such as IL-10 and TGF-β, and defects in functions of $CD8^+$ $CD28^-$ nTregs are associated with the pathogenesis of Rheumatoid arthritis (RA)[70]. $CD8^+$ $CD28^-$ nTregs can also induce the up-regulation of inhibitory receptors such as ILT3/4 on APCs, rendering them tolerogenic. These tolerogenic APCs can in turn induce Tregs or T cell anergy[71].
In addition to $CD8^+$ nTregs, induced $CD8^+$ Tregs ($CD8^+$ iTregs) have been also identified. Recent studies showed that $CD8^+$ Tregs expressing FoxP3 transcription are induced during graft-versus-host disease (GVHD) in mice[72,73]. In GVHD, these $CD8^+$ $FoxP3^+$ iTregs express surface markers commonly found on $CD4^+$ Tregs such as GITR, CTLA-4, and they constitute a significant percentage of the entire Treg population in mice undergoing GVHD. Moreover, $CD8^+$ iTregs prevent increased GVHD mortality, even in the complete absence of $CD4^+$ Tregs. Another subset of $CD8^+$ iTregs is induced upon Mycobacterium tuberculosis bacillus Calmette-Guerin infection. These $CD8^+$ iTregs express LAG-3 and FoxP3

and suppress T cell functions through the secretion of CC chemokine ligand 4 (CCL4)[74]. $CD8^+$ $FoxP3^+$ iTregs can also be generated upon ex vivo restimulation with antigen in presence of TGF-β[75].

Given the important functions of Tregs in immune homeostasis, defects in functions and numbers of Tregs are often implicated in the pathogenesis of auto-immune and inflammatory diseases. It has been demonstrated that selective deletion of $FoxP3^+$ Tregs in mice led to severe systemic autoimmunity, leading mice to death within 10 days. The rare fatal autoimmune disease IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) and the murine counterpart scurfy, which are caused by mutation in FoxP3 gene, further support the importance of Tregs in the immune regulation[76-78]. Therefore, strategiesaddressed to expand and enhance activity of Tregs in order to reduce or completely resolve autoimmune disorders have been object of extensive study. In particular, Tr1 were generated in diabetic mice that were treated with a combination of rapamycin and IL-10[62]. In addition, the isolation of Tregs from patients with RA, SLE, Type 1 diabetes and MS and their expansion in vitro with α-CD3 and α-CD28 has been also investigated as treatment of these auto-immune disorders[79-81]. To conclude, Tregs are essential T cells in maintaining immune homeostasis, thus preventing immune-pathologies such as autoimmune diseases, allergy and allograft rejection.

Cancer Immunoediting

Besides its crucial role in protecting the body from invading microorganisms, the immune system can prevent tumor development by recognizing and eliminating the nascent transformed cells that express modified antigens compared to the normal tissues. The idea that the immune system could control tumor development and neoplastic disease was conceived at the beginning of twentieth century and incorporated in the hypothesis of "cancer immunosurveillance" of Burnet and Thomas in 1950s[82]. However, this hypothesis was shortly abandoned afterwards because no experimental evidence could support this concept. Many years later, experiments conducted in mice and clinical studies in humans not only demonstrated the existence of cancer immunosurveillance, but they further

expanded the concept indicating that the immune system, besides eliminating tumors, is also responsible of sculpting the immunogenic phenotypes of tumors that eventually develop in immunocompetent hosts[83,84]. More specifically, during tumor elimination, the immune system leads to the selection of tumor cell variants that can better survive in an immunologically intact environment, promoting tumor development and malignant disease. Accordingly, over the past years the concept of "cancer immunosurveillance" was replaced by the term of "cancer immunoediting" to better describe the observed process[85]. Cancer immunoediting is mainly divided in three phases, also known as the three Es: elimination, equilibrium and escape[86] (Fig. 2).

The elimination phase includes the original concept of cancer immunosurveillance. During tumor growth, inflammatory signals derived from the disruption of the surrounding tissues induce the recruitment of innate cells such as DCs, macrophages, and NK cells[87]. The recognition of tumor associated ligands such as MICA/B (MHC-I-chain-related A/B) in humans and RAE-1 (retinoic acid inducible early transcript-1) and H60 in mice by the NKG2D receptor on NK cells induces the production and release of IFN-γ, which induces direct killing of tumor cells and stimulates the phagocytic activity of macrophages and DCs[88-90]. Moreover, IFN-γ also induces the production of chemokines such as CXCL10 and CXCL9 by the tumor and the surrounding normal tissues. These chemokines recruit more NK cells and macrophage to the tumor site. Necrotic and apoptotic tumor cells are ingested by DCs, which consequently migrate to the tumor-draining lymph nodes in order to induce tumor- specific $CD4^+$ and $CD8^+$ T cells. In particular, tumor-antigen presenting DCs differentiate $CD8^+$ T cells into CTLs and $CD4^+$ T helper into Th1 producing IFN-γ that in turn facilitate the development of CTLs. Upon their activation in tumor-draining lymph nodes, CTLs and Th1 home to the tumor site, where CTLs directly destroy the antigen-bearing tumor cells, while Th1 help the other cells of the immune system to eliminate the tumor by secreting IFN-γ[91,92]. Tumor cell variants that survived the elimination phase enter the equilibrium phase. Here, lymphocytes and IFN-γ exert selection pressure on tumor cells in order to contain the growing of many unstable and mutating cells. During this phase, new tumor variants arise with different mutations that make them resistant to the immune attack and enable them to enter the escape phase.

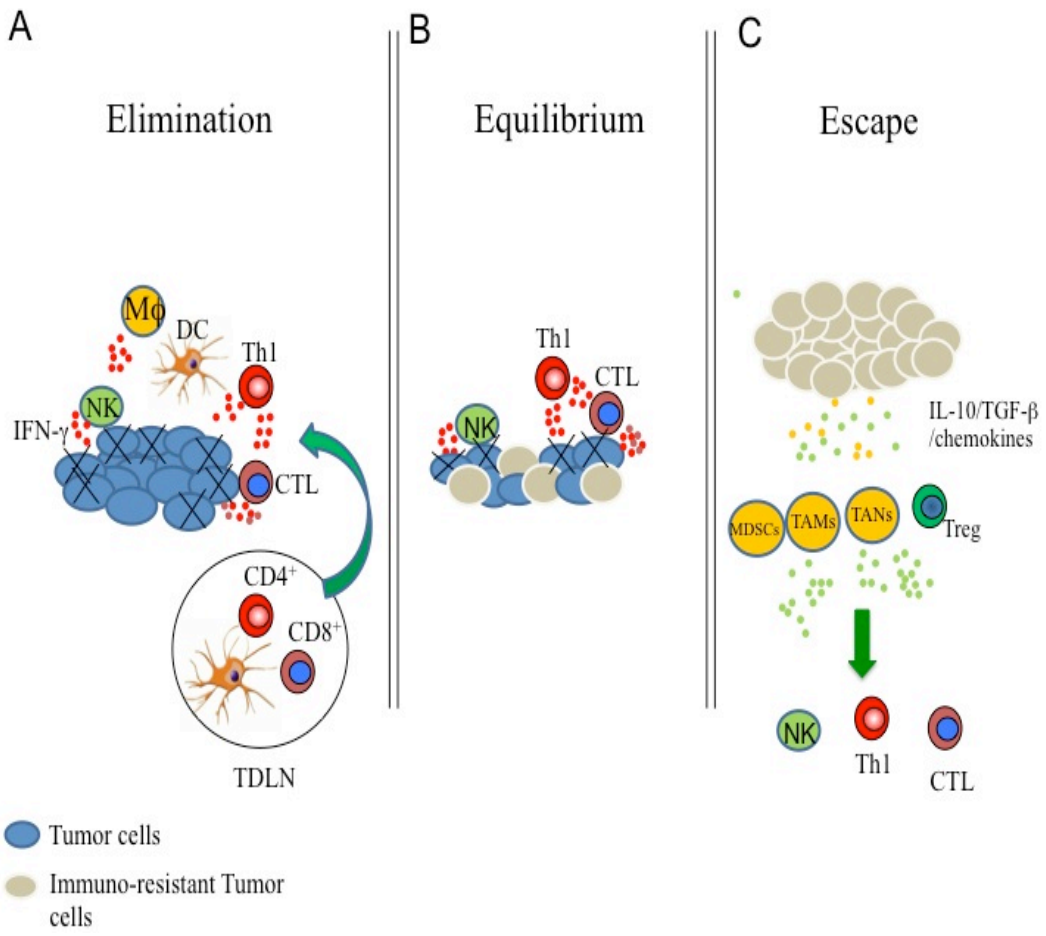

Figure 2: the three Es of cancer immunoediting. Cancer immunoediting is divided in three phases: elimination (A), equilibrium (B) and escape (C).(A) During tumor development, NK cells, macrophages and DCs are recruited to the tumor site. Following their recruitment, NK cells produce IFN-γ, which leads to tumor cells death and stimulates phagocytic activities of macrophages and DCs. Tumor cell death by IFN-γ produces tumor cell debris that is ingested by DCs, which migrate to the tumor-draining lymph nodes to activate and differentiate $CD8^+$ and $CD4^+$ into CTLs and Th1, respectively. Upon their activation, CTLs and Th1 home to the tumor site, where CTLs directly destroy the transformed cells, while Th1 produce IFN-γ potentiating the properties of the other tumor-infiltrating immune cells. b) Equilibrium represents the process by which the immune system selects and/or promotes the generation of tumor cell variants with increasing abilities to survive immune attack. c) Escape is the process where tumor cell variants that are insensitive to immune suppression in the equilibrium phase expand in an uncontrolled manner. The secretion of chemokines and anti-inflammatory cytokines by growing tumor creates an immunosuppressive tolerogenic/immunosuppressive tumor microenvironment enriched of tumor-associated macrophages (TAMs), tumor- associated Neutrophils (TANs), myeloid-derived suppressor cells (MDSCs) and $CD4^+/CD8^+$ Tregs. These cells secrete in turn anti-inflammatory cytokines such as IL-10 and TGF-β to inhibit effector functions of NK cells, Th1 and CTLs. Tregs also inhibit effector immunity in a cell-contact manner.

In the escape phase, surviving tumors are insensitive to immune suppression and begin to expand in an uncontrolled manner, which leads to the tumor formation and malignancy. During their growing and progression, tumors are generally infiltrated by immune cells. However, the tumor-infiltrating immune cells not only fail to exercise anti-tumor effector functions, but they can favor tumor progression and survival[93]. This is mainly due to the secretion of chemokines including chemokine (C-C motif) ligand 2 (CCL2), colony stimulating factor-1 (CSF1) and chemokine (C-X-C motif) ligand 12 (CXCL12)[94] by tumor cells, which recruit macrophages and neutrophils and DCs to the tumor site. Tumor cells also produce cytokines such as IL-10 and TGF-β, which polarize these tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) towards an anti-inflammatory phenotype[95]. TAMs and TANs promote key processes of tumor progression including angiogenesis, invasion and metastasis, and they downregulate and interfere with functions of other effector immune cells by secreting IL-10 and TGF-β[96,97]. Tumors also recruit a variety of immature myeloid cells, also known as myeloid-derived suppressor cells (MDSCs), which exert pro-tumor activities[98].

The tumor microenvironment is also enriched in $CD4^+$ and $CD8^+$ Tregs and their high accumulation in the microenvironment has been associated with a worse prognosis of many tumors such as human ovarian cancer, melanoma, pancreatic cancer and breast cancer[99-103]. In addition to the tumor itself, high number of nTregs such as $CD4^+$ $CD25^{hi}$ $Foxp3^+$ and $CD8^+$ $CD28^-$ Tregs accumulate in the peripheral blood of patients with cancer[104,105]. Besides recruiting nTregs via chemokines, the tumor microenvironment also promotes the expansion of nTregs as well as in situ generation of iTregs. The latter is due to the action of IL-10 and TGF-β that are produced by tumor cells and MDSCs[106]. Within the tumor, the recruited and expanded nTregs suppress the functions of effector immune cells together with iTregs via both a CTLA4-dependent mechanism and the secretion of anti-inflammatory cytokines. Tregs thus play a crucial role in tumor escape from the immune system. Treatment of mice with anti-CD25 antibodies alone or together with anti-CTLA-4 antibodies significantly inhibited the development of many types of tumors[107,108]. The anti-tumor activity of tumor-specific $CD8^+$ T cells transferred into B16 melanoma-bearing mice was

impaired when $CD25^-$ $CD4^+$ T cells were co-administrated, which further supports a role for Tregs in hindering anti-tumor immune responses[109]. Therefore, strategies directed to deplete or reduce the activity of tumor- associated Tregs can improve the effectiveness of cancer immunotherapy.

Aberrant glycosylation on poorly immunogenic tumors

Different mutations occur in each tumor cell during tumor growth, resulting in intra-tumoral heterogeneity in phenotypic features such as cellular morphology, gene expression, metabolism and immunogenic potential[110]. This tumor heterogeneity is also reflected in changes in glycosylation of tumor cells. Glycosylation is the enzymatic process that produces glycosidic linkages of glycans to proteins and lipids, and it is essential for cell viability and functionality[111]. Glycan chains of glycoproteins can be classified in N-glycans, and O-glycans according to the linkage to glycoproteins. N-Glycans are linked to the asparagines (Asn) of the underlying glycoproteins, while O-glycans are linked to serin/ threonine residues[112]. N- glycans and O-glycans are aberrantly expressed in tumors[113]. The most common tumor- associated glycans are the O-glycans Tn antigens, which are only composed of N-acetyl-D-galactosamine (GalNAc) with a glycosidic α-linkage to serine/threonine residues in glycoproteins[114]. The Tn antigen is normally modified in the Golgi apparatus of the cell by T- synthase which catalyzes the addition of galactose (Gal) to the Tn-antigen, leading thus to the estension of the O-linked glycan chains. The expression of active T-synthase is uniquely dependent on the molecular chaperone Cosmc. Accordingly, mutations and loss of Cosmc found in tumor cells lead to the expression of the Tn-antigen[115]. Tn antigens have been found particularly overexpressed on MUC-1 in adenocarcinomas such as breast-, pancreas- and colon carcinoma[116]. It has been described that Tn-antigen on tumor-associated MUC-1 is recognized by DCs via interaction with the macrophage galactose type lectin (MGL)[116]. MGL is a C-type lectin receptor (CLR) mainly expressed on macrophages and DCs in humans, rats and mice[117,118]. In humans, MGL is involved in the recognition of GalNAc-containing antigens, which leads to the antigen internalization, processing and presentation[119]. Since CLRs are known not to influence DC

maturation, in human the internalization of tumor- associated MUC-1 by DCs via MGL is likely to induce tolerance and tumor progression[120].

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Tumors also highly express Tn-antigens that are over-capped by sialic acids (sialyl-Tn). Sialic acids are also present on other glycans or glycoconjugates whose high or aberrant expression often correlate with tumor progression. These include Sialyl Lewis X or Sialyl Lewis A ($SLe^{X/A}$) glycan and a particular class of glycosphingolipids: the gangliosides[121]. In tumors, the overexpression of sialic acids is mainly due to the upregulation of the beta-galactoside α2- 6-sialyltransferase 1 (ST6Gal-1), which is a transporter in the Golgi membrane and responsible for adding sialic acid onto nascent glycoconjugates[122].

As in the case of Tn-antigen, DCs also express receptors that specifically recognize sialic acids: these receptors include Selectins and Sialic-acid binding lectins (Siglecs). Siglecs are modulators of the immune system negatively regulating APC function upon binding sialic acids[123]. Nevertheless, it is not clear whether sialic acids on tumors can dampen the function of tumor-associated DCs through the interaction with Siglecs, inducing thus tolerance and tumor progression. So far, it has been described that sialic acids that are highly expressed on tumors contribute in tumor progression and metastasis especially because of their biophysical properties. More specifically, the high negative charge of sialic acids promote the detachment of tumor cells from the primary tumor through charge repulsion, facilitating thus the dissemination of tumor cells through the bloodstream. Once in the bloodstream, tumor cells can interact with selectins on platelets and endothelial cells via the SLe^{x} or SLe^{A} structures. This binding arrests tumor cells and enable them to enter into distant endothelial sites to form metastases[124-126].

Sialic acids

Sialic acids are a widely family of carbohydrates mainly distributed in nature as the outermost sugars present on glycoproteins and glycosphingolipids. Sialic acids consist of monosaccharides with a nine-carbon backbone[127,128]. According to the structural modification of the monosaccharide, different types of sialic acids can be found in nature. The diversity of sialic acids is also provided by how these sugars are attached to the underlying glycan. In particular, the linkage of Carbon in position 2 (C-2) of sialic acid to C-3 of the underlying galactose residue generate

α2-3-linked sialic acid, while binding of C-2 of sialic acid to C-6 of galactose or N-acetylgalactosamine residues induces α2-6-linked sialic acid. In nature, the glycan chains can also be disialylated or polysialylated. In this case, sialic acid is linked to C-8 of another sialic acid to form α2-8-linked sialic acid. This diversity in linkage mainly determines different binding specificity of sialic acids for several carbohydrate binding receptors, including Siglecs.

Given their terminal position, sialic acids exert several biological and physiological functions[129](Fig. 3). Due to their electronegative charge, sialic acids are implicated in the stabilization of cells, molecules and in regulation of interaction with the environment. The high expression of sialic acids on the membrane of erythrocytes contributes to the fluidity of blood by avoiding their aggregation through charge repulsion. Their negative charge also enhances viscosity of mucins by attracting water. As terminal monosaccharide, sialic acids contribute in the protection of self cells and molecules by masking the underlying galactose (Gal) or GalNAc of the glycan chain from the attack by macrophages and granulocytes via macrophage galactose lectin (MGL), avoiding thus cell or molecule degradation. In addition, sialic acids inhibit the activation of the complement cascade by binding Factor H, which is a complement cascade inhibitor.

Sialic acids are recognized by different lectins found in animals, plants and microorganisms[130]. Some sialic acid-binding lectins have been identified for their capacity to agglutinate animal cells and precipitate glycoconjugates. These include viral hemagglutinin, and plant lectins such as *Maackia Amurensis* agglutinin (MAA) and *Sambucus Nigra* agglutinin (SNA). In particular, the interaction of hemagglutinin with sialic acids present on the host cell is responsible for the attachment of influenza viruses to the host cell[131,132]. In contrast, human immune cells express sialic acid binding lectins that include Selectins and Siglecs. Selectins are divided in three subsets according to the site/cell type of expression: E- selectin is expressed on endothelial cells, L-selectin in leukocytes, while P-selectins are primarily found on platelets and endothelial cells. These selectins prevalently bind SLe^X; the interaction of SLe^X present on leucocytes with E-selectins initiates leukocytes rolling on endothelial surfaces leading to leukocyte extravasation to the site of infection[133,134].

Siglecs

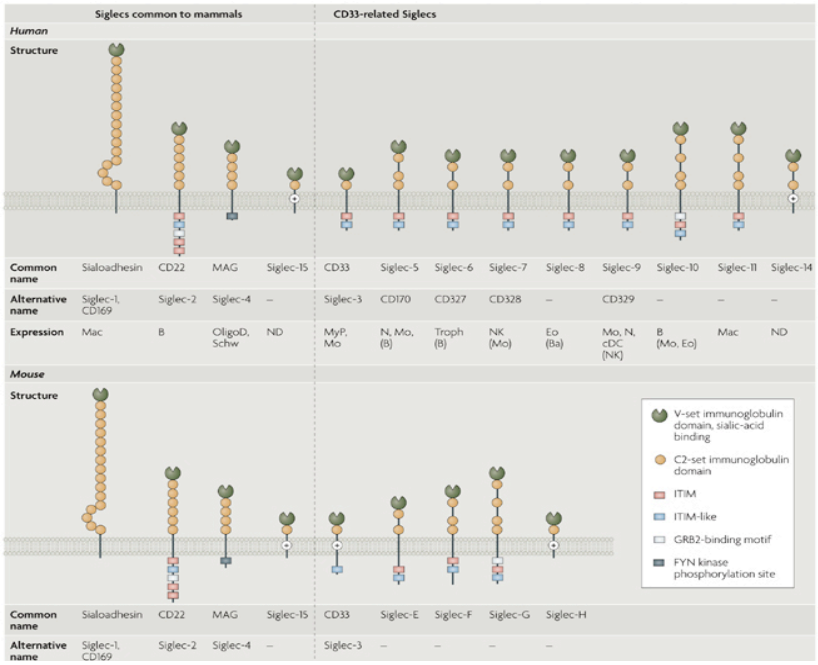
Siglecs are members of the immunoglobulin-type lectins (I-type-lectins), and they are mainly expressed on immune cells, where they act as negative regulators of immune processes including cellular activation and proliferation, apoptosis, cytokine secretion, and endocytosis[135].

Structurally, Siglecs contain sialic acid binding amino-terminal V-set immunoglobulin (Ig) domain and variable numbers of C2-set Ig domains. Siglecs can be divided into two subgroups: the first subgroup comprise the evolutionary well conserved Siglecs including Sialoadhesin 1 (also known as Siglec-1), CD22 (also known as Siglec-2), Myelin-associated glycoprotein (MAG, known as Siglec-4) and Siglec-15. The second subgroup is represented by CD33-related Siglecs (CD33rSiglecs) such as CD33 (also known as Siglec-3), Siglec-5, -6, -7, -8,-10, -11,-14 and 16[136] (Fig. 3).

Humans have ten CD33rSiglecs, while mice have only five, namely CD33/Siglec-3, Siglec-E, -F, -G, -H. Murine Siglecs have functional paralogs in humans that have been generated by convergent evolution. For example, 3-Ig-domain containing Siglec-E shows sequence similarity with Siglec-7,-8 and -9, which arose from gene duplication, whereas Siglec-F has been proposed to be the ortholog of human Siglec-5. Singles bind different linked silica acids and each Sigel has a unique specificity profile for binding different sialoside structures found in mammalian glycoproteins and glycolipids (Fig. 3).

Siglecs are present on various subsets of leukocytes in a cell-type-specific manner. The only exception is Siglec-4 or MAG, which is exclusively expressed in the nervous system. Sialoadhesin is expressed on macrophages[137] and murine DCs[138]. CD22 expression is only found on B cells[139], while Siglec-15 expression is restricted to monocytes, macrophages and DCs[140]. Among murine CD33r-Siglecs, CD33 is mainly expressed in granulocytes[141]; Siglec-E is primarily expressed in monocytes, neutrophils and DCs; Siglec-F expression is restricted to eosinophils[142]; Siglec-G is expressed in B cells[143], while Siglec-H is mainly expressed in plasmacytoid DCs[144]. However, certain CD33rSiglecs are broadly present on multiple cell types. For example, expression of Siglec-7 is reported on various subsets of leukocytes such as NK, monocytes, DCs and $CD8^+$ T cells[145] (Fig.3).

A



B

	Human					Mouse		
	CD22	Siglec-7	Siglec-8	Siglec-9	Siglec-10	CD22	Siglec-E	Siglec-F
α3 β4	0	+	+	+	+	0	++	++
α6 β4	+++	+	+	+	++	+	++	+/–
α6 β4	+++	+	+	+	+++	+++	+	0
α8 α3 β4	0	+++	+	0	0	0	++	0
6S α3 β4 α3	ND	++	+++	0	0	0	++	+++
6S α3 β4 α3	ND	+	0	+++	0	0	+	0

Neu5Ac Galactose GlcNAc Neu5Gc Fucose S Sulphate

Figure 3: Structure and expression of the Siglec family. A, Siglec expression in humans (upper part) and mouse (lower part). Siglecs are divided in two distinct subgroups represented by the evolutionary conserved Siglecs (left side of the dotted lines) and the rapidly changing CD33rSiglecs (right side of the dotted lines). Alternative names and expression of each siglec are indicated. Mac, macrophage; B, B cells; OligoD, oligodendrocytes; ND, not determined; MyP, myeloid progenitors; Mo, monocytes; N, neutrophils; Troph, trophoblasts; NK, natural killer cells; Eo, eosinophils; Ba, basophils,; cDC, conventional DCs. B, binding affinities of Human and mouse Siglecs for different selected sialoside sequences found in mammalian glycoproteins and glycolipids. +++, strong binding; ++, moderate binding; +, low binding; +/-, detectable binding; 0, no detectable binding. GlcNAc, N-acetylglucosamine; Neu5Gc, N- glycolylneuraminic acid; ND, not determined. Figures adapted from Paul R. Crocker, James C. Paulson and Aijt Varki, *Nat Rev Immunol.*, 2007.

Given the high local concentration of sialic acids on surface of immune cells, Siglecs usually bind the sialoside structures present on the same cells, also known as “cis” binding. The interactions with cis ligands can dominate over interactions with sialic acids on other cells (“trans” binding), in modulating the biological activities of Siglecs[146]. The cis binding of Siglecs to sialic acids seems to prevent random activation of Siglec-containing immune cells. For instance, the cis interaction of CD22 on B cells with α2-6-linked sialic acids prevents autoimmunity by dampening B cell autoreactivity[147]. However, high affinity sialoside structures can outcompete the cis ligands for binding Siglecs. High-affinity synthetic sialoside probes can outcompete cis ligands for binding to CD22 on B cells, indicating that cis ligands downregulate, but do not preclude, binding of ligands in trans[148].

Several human and murine Siglecs contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their intracellular portion, which trigger a signaling cascade that negatively regulates activation of immune cells. More specifically, the interaction of ITIM-containing Siglecs with sialic acids induces tyrosine phosporylation of the ITIM domain by Src-family kinases followed by the recruitment of Src homology 2 domain-containing inositol polyphosphate 5’ phospatases (SHIP) or Src homology 2 domain-containing protein phosphatases (SHP) that subsequently leads to the inhibition of imunoreceptor tyrosine-based activation motif (ITAM)-dependent signaling[149]. The best understood ITIM-bearing Siglec is CD22, which regulates B cell signaling, homeostasis and survival of B cells[150]. Certain CD33rSiglecs such as Siglec-14, -15, and -16 lack ITIM motifs, and instead interact via a positive charged residue in their transmembrane domain with ITAM-containing DAP- 12, which can induce both activating or inhibitory signaling cascades.

Given the modulatory properties of Siglecs, sialic acids can negatively affect the activation of the immune response through the interaction with Siglecs. Certain human pathogens present high levels of sialic acids or have evolved to coat themselves with host-derived sialic acids in a process called molecular mimicry to interact with Siglecs on immune cells and consequently dampen the immune response. It has been reported that the sialylated capsular polysaccharide of group B streptococcus (GPS) engages neutrophil Siglec-9, dampening neutrophil

responses in a Siglec-9-dependent manner[151], while sialic acids on Pseudomonas Aeruginosa bind Siglec-7 and Siglec-9 on NK cells and monocytes, herewith dampening their functions[152]. Furthermore, it has been demonstrated that sialylated lypoligosaccharides (LOS) of bacterium C. jejuni can modulate the ability of DCs to polarize naive $CD4^+$ T cells inducing Th1 and Th2 differentiation by binding Siglec-1 and Siglec-7, respectively[153]. However, it is unclear how Siglec-mediated dampening influences $CD4^+$ T cell polarization and/or $CD8^+$ T cell differentiation. Moreover, it is not clear whether sialic acids can dampen anti-tumor immune responses by interacting with Siglecs expressed on tumor-infiltrating immune cells.

Scope of the thesis

The inability of the immune system to completely eradicate tumors is mainly due to a tolerogenic tumor microenvironment. It has been shown that poorly immunogenic tumors tend to aberrantly express sialic acids. However, a direct relation between tumor hyper- sialylation and poor immunogenicity has not been defined. In **chapter 2**, we reduced the sialylation of the highly sialylated murine melanoma B16-OVA by gene knockdown to ascertain the effects of hyper-sialylation on anti-tumor immunity. We demonstrate that a reduction in the levels of sialic acids on melanoma delayed tumor growth in tumor-bearing mice and was associated with lower frequencies of intra-tumoral $CD4^+$ and $CD8^+$ Tregs and increased numbers of infiltrating NK cells.

The observation that levels of sialic acids on tumor directly correlated with numbers of tumor-infiltrating Tregs led us to investigate in more detail the role of sialylated antigens in the activation and differentiation of T cells. In **chapter 3**, we showed that DC-mediated uptake of Ovalbumin (OVA) modified with sialic acids (Sia-OVA) strongly induced the differentiation of naive OVA-responsive $CD4^+$ T cells into $FoxP3^+$ $CD4^+$ T cells while preventing induction of IFN-γ^+ $CD4^+$ T cells in vitro and *in vivo*. Moreover, we showed that the absence of Siglec-E on DCs significant reduced binding and uptake of Sialic acid-modified OVA as well as totally abolished their capacity to generate $CD4^+$ Tregs. In **chapter 4**, we observed that the uptake of Sia-OVA by DCs potently inhibited

the differentiation of OVA-specific cytotoxic $CD8^+$ T cells and instead induced $CD8^+$ $FoxP3^+$ T cells. Under inflammatory conditions, $CD8^+$ $FoxP3^+$ T cells were not generated, which is in contrast to our findings on $CD4^+$ $FoxP3^+$ T cells.

In **chapter 5**, we aimed at elucidating the mechanisms enabling Sia-OVA-loaded DCs to alter the activation program of OVA-responsive T cells. Preliminary data revealed a cell-contact restricted inhibition of effector $CD4^+$ T cell response by Sia-OVA-loaded DCs. Moreover, gene expression profiling of Sia-OVA-loaded DCs revealed the overexpression of genes that can be involved in the observed effects on T cell activation.

Finally, in **chapter 6** the results described in this thesis are discussed and put into perspective of therapeutic implications of sialic acids in the treatment of tumors and auto-immune disorders.

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above: Coppo di Marcovaldo; *detail of the Hell scene* (1265-70). Baptistery of Florence cathedral.

Chapter 2

Tumor sialylation negatively instructs NK and T cell-mediated anti-tumor responses while promoting tumor associated-Tregs

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Manuscript submitted

Abstract

Tumors present a tolerogenic microenvironment, which allows escape from tumor-specific effector cells. The contribution of high levels of sialic acids (Sias) on the tumor surface to immune escape is unclear. Here we demonstrate that genetically engineered Sia^{low} B16 tumors grow slower than wild-type Sia^{high} B16 tumors. Higher frequencies of IFN-γ-producing effector T cells were detected in the tumor-microenvironment of Sia^{low} than of Sia^{high} tumors. Moreover this was paralleled by a 50% reduction in $CD4^{+}$ $FoxP3^{+}$regulatory T cell (Treg) frequencies in Sia^{low} tumors. The enhanced effector T-cell response we observed at the end- stage was preceded by an enhanced influx of Natural Killer (NK) cells in developing tumors. The reduced presence of Sias on tumors evoked NK cell activation and production of IFN-γ, which made the micro-milieu of Sia^{low} tumors less tolerogenic. In the absence of NK cells the influx of IFN-γ producing effector T cells in Sia^{low} tumors was absent, unveiling the importance of NK cell activation as initiating step in anti-tumor immunity in Sia^{low} tumors. In conclusion, tumor hyper-sialylation orchestrates immune escape at the level of effector T cells, NK cell activation and influx of Tregs within the tumor microenvironment and herewith tumor progression. Reducing sialylation may provide a therapeutic option to render tumors permissive to immune attack.

Introduction

Innate immune cells play a crucial role in the immune response against tumors. Particularly, dendritic cells (DCs), macrophages and NK cells are involved in the first phase of the process known as cancer immunoediting, where these cells detect the presence of a developing tumor and coordinate as well as co-operate with the adaptive immune system to eradicate the tumor [1]. DCs present tumor antigens on MHC class I molecules, which together with costimulatory and pro-inflammatory cytokines drives $CD8^+$ T cell expansion and concurrent acquisition of effector functions such as cytolytic activity and/or production of cytokines (i.e. interferon (IFN)-γ and tumor necrosis factor (TNF) [2]. Activation of tumor antigen-specific $CD4^+$ T cells by presentation of tumor antigens in MHC class II molecules is important as they influence induction of long-term memory $CD8^+$ T cells. Additionally, tumor-specific $CD4^+$ T cells contribute to the eradication of tumors, either via direct killing of tumor cells [3] or via the activation of macrophages at tumor sites [4]. Next to cytotoxic T cells, also NK cells have the capacity to lyse tumor cells. Yet, in contrast to T cells, NK cells represent the first line of defense against transformed cells. Low or absent expression of MHC-I triggers NK cell effector functions, which include release of IFN-γ and cytotoxic granules and induction of apoptosis of target cells. High frequencies of intra-tumoral NK cells have been associated with good prognosis of patients [5].

Hence, alterations in frequency and function of innate cells contribute to tumor immune escape and consequent tumor overgrowth. Indeed, DCs and macrophages that are present in the tumor microenvironment are characterized by an anti-inflammatory or tolerogenic phenotype [6]. Furthermore, high numbers of both natural as well as induced regulatory T cells (nTregs and iTregs) infiltrate into the tumor site [7, 8]. Both, Tregs and tolerogenic DCs and macrophages block anti-tumor immunity by complicating effector T cell generation. Additionally, Tregs prevent effector T cell infiltration into the tumor by promoting alterations in the expression of intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM), involved in adhesion and transmigration of T cells, on tumor endothelial cells tumor vasculature [9].

Tumor cells display aberrant glycosylation. In particular, increased levels of Sialic acids (Sias) are often correlated with tumor invasion and poor prognosis of malignancies [10, 11]. Sialic acids (Sias) are the outermost monosaccharides present on glycan chains of glycoconjugates such as glycoproteins and glycolipids. These sugars are attached to the underlying glycans, with different linkages such as α2-3-, α2-6- and α2-8-linkages [10]. Given their terminal position, Sias are the recognition elements for Sia-binding Ig-like lectins (Siglecs) and selectins. Binding of Sias by these receptors influences many physiological processes, such as cell growth, differentiation and motility [10]. In tumors, the levels of Sias are increased mainly due to enhanced expression and activity of the beta-galactoside α2-6- sialyltransferase 1 (ST6Gal-1), which is responsible for adding Sias onto nascent glycoconjugates [12]. The negative charge of Sias might cause the dissociation of high Sias expressing tumor cells from the primary tumor, leading to the dissemination of tumor cells through the bloodstream. Subsequent engagement of Sia-binding selectins expressed by platelets and endothelial cells arrests tumor cell migration and facilitates their invasion of underlying tissues and formation of metastasis [13-15].

Siglecs show a restricted expression pattern and are predominantly found on innate immune cells [16]. On these cells, siglecs function as endocytic receptors and regulate their activation, proliferation and cytokine secretion. The CD33-related siglecs and CD22 (Siglec-2) can act as negative regulators of immune responses via the expression of membrane-proximal Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in their cytoplasmic tails [16]. Following phosphorylation by Src-family kinases, the ITIMs of siglecs recruit phosphatases (e.g., SHP-1/2). In turn, these SHPs can de-activate various kinases acting downstream of other receptors, herewith effectively down-regulating their functions [17]. Indeed, CD22/ Siglec-2 and Siglec-G co-operate on the B-cell surface to inhibit B-cell receptor signalling [18].

Campylobacter jejuni and Neisseria meningitidis have been shown to negatively affect human APC function and consequently subvert immune responses. A common feature of these pathogens is high expression of Sia on their surface [19-24]. Indeed, increased sialylation of pathogen-derived Sia-bearing gangliosides prevented the activation of DCs, as indicated by a reduction in co-stimulatory

molecule upregulation and secretion of IL-6 and IL-12, which together resulted in reduced Th1 generation [20, 24]. Sias on Leishmania spp. negatively affected nitric oxide, IL-12 and IFN-γ production by human macrophages, inhibiting host responses and ensuring increased survival of the pathogen [15, 25]. Thus, anti-inflammatory effects of Sia-bearing pathogens on antigen presenting cell function have been shown. By contrast, effects on T-cell responses are hardly studied.

In view of the important role of the innate immune system in tumor immune surveillance we investigated whether high levels of Sias on the tumor surface promote tumor immune evasion by paralyzing immune effector functions.

Materials and Methods

Cells and lentiviral transduction

B16-OVA (murine melanoma; kindly provided by Dr. T. Schumacher, National Cancer Institute, Amsterdam, The Netherlands) were cultured in DMEM supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin (BioWhittaker, Walkersville, MD).

Sia^{low} B16-OVA (hereafter named $B16^{SLC35A1}$) were generated by transducing tumor cells with the pLKO.1 lentiviral vector containing shRNA targeting the SLC35A1 gene. As a control, B16-OVA cells were transduced with pLKO.1 containing non-target shRNA (designated as $B16^{scrambled}$). Lentiviral vectors were produced as described earlier [26]. In short, HEK293T cells were co-transfected with pLKO.1-shRNA and the packaging plasmids (pVSV-G, pMDL and pRev-RRE) in the presence of calcium phosphate. One day later, medium was replaced by serum-free medium and culture supernatant was harvested another day later. B16-OVA cells were seeded in a 6-well plate format at 10^5 cells/well and transduced with a fixed amount of lentiviral vector when 80% confluent. The day following infection, target cells were selected with 1 μg/ml puromycin.

Tumor experiments

C57BL/6 mice were purchased from Charles River Laboratories and used at 8-12 weeks of age. For *in vivo* studies, $1x10^5$ tumor cells were inoculated subcutaneously into the flank of mice. Mice were sacrificed when tumors reached a diameter of 1.5 cm. Tumor volume was calculated according the formula: $length \times width^2 \times \pi/6$. After sacrifice, tumors, spleens and lymph nodes were removed for analysis by flow cytometry. To deplete NK cells, mice were injected i.p. with anti-NK1.1 antibodies (PK136; mouse IgG2a; 0.2 mg) on days -3, 0, 4, 8 from the start of tumor challenge. From day 8 on, anti-NK1.1 antibodies were injected once a week. All experiments were approved by the Animal Experiments Committee of the VUmc, Amsterdam.

Tumor-infiltrating lymphocytes, spleens and tumor-draining lymph nodes

For isolation of tumor-infiltrating lymphocytes, tumors were removed from C57BL/6 mice, cut into grain size pieces and incubated in HE medium (RPMI medium with 10% FCS, 10 mM EDTA, 20 mM HEPES, 50 U/ml penicillin, 50 µg/ml streptomycin) supplemented with 1 WU/ml Liberase-TL (Roche Diagnostics GmbH, Manheim, Germany) and 50 µg/ml DNase I (Roche) for 30 min at 37°C. Red blood cells were lysed with ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) and undigested material was removed by filtration. Subsequently, tumor-infiltrating lymphocytes were purified by ficoll gradient with Lymphoprep (Axis-Shield, UK).

Tumor-draining lymph nodes and spleens were passed through a 100 µm cell strainer (BD Falcon, NJ, USA) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, CA, USA) to generate single cell suspensions. Red blood cells were lysed with ACK lysis buffer. Subsequently, the presence of different immune cells was analyzed upon staining with specific antibodies and flow cytometry. Alternatively,

GAPDH F	GAC AAC TCA TCA AGA TTG TCA GCA
GAPDH R	TTC ATG AGC CCT TCC ACA ATG
IL 10 F	GGA CAA CAT ACT GCT AAC CG
IL 10 R	GGG G=CAT CAC TTC TAC CAG
TGFβ-1 F	GCT GAA CCA AGG AGA CGG AAT A
TGFβ-1 R	GGG CTG ATC CCG TTG ATT T
IP-10 F	GAC GGT CCG CTG AAC TG
IP-10 R	GCT TCC CTA TAT GGC CTC ATT
IFNγ-R F	GCTTTGACGAGCACTGAGGA
IFNγ-R R	CCAGCATACGACAGGGTTCA

cells were re-stimulated with 30 µg/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) for detection of IFN-γ and IL-10 in cell supernatants by ELISA using specific antibody pairs and following manufacturer's instructions (eBiosciences, CA, USA).

Co-cultures of tumor cells and DCs or NK cells

Effects of tumor expressed Sia on DC phenotype was examined in a co-culture of DCs with $B16^{SLC35A1}$ or $B16^{scrambled}$. The next day, DCs were harvested and expression of MHC and costimulatory molecules on $CD11c^{+}$ cells was analysed by flow cytometry. Supernatants were analyzed for the presence of cytokines using the cytometric bead array (CBA; BD Biosciences). To examine effects of sialylated tumorantigens on NK cell function, either total splenocytes or NK-enriched splenocytes derived from naive C57BL/6 mice were co-cultured at indicated E:T ratio with $B16^{SLC35A1}$ or $B16^{scrambled}$ tumor cells. Four hours after co-culture, the amount of IFN-γ in the supernatant was analysed by ELISA.

To determine effects of NK-derived IFN-γ on tumor-expressed chemokines and cytokines, $B16^{SLC35A1}$ and $B16^{scrambled}$ were cultured in the presence or absence of 250 pg/ml IFN-γ for 6 hours and expression of chemokines and cytokines was measured by qRT-PCR.

Analysis of cell cycle, adhesion and motility

For cell cycle analysis by DNA content, tumor cells were collected and fixed in 70% ethanol o/n at 4°C. After washing, tumor cells were resuspended in PBS containing 10 µg/ml Propidium Iodide and 200 µg/ml RNase A and analyzed by flow cytometry.

For cell adhesion analysis, Nunc-immuno Maxisorp plates (Sigma-Aldrich) were coated o/n with 50 ml matrigel (BD Biosciences) 1:25 in PBS buffer. After washing with TSM buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$), plates were incubated for 30 min at 37°C with TSM containing 0,5% BSA to block non-specific binding. CFSE- labeled tumor cells were allowed to adhere for 30 min at 37°C, and non-adherent cells were removed by gently

washing plates three times with pre-warmed TSM/0.5% BSA. Subsequently, bound tumor cells were lysed using a 50 mM Tris/0,1% SDS buffer and fluorescence was detected using a CytoFluor (Applied Biosystems, CA, USA).
A motility or scratch assay was performed as previously described [27]. Briefly, B16-OVA cells were seeded in 24-wells plate and when confluent a p200 pipet tip was used to create a scratch in the cell monolayer. Images of gap closure by cells were captured every 3 hours by digital microscope camera (Leica DFC420, Leica Microsystems, Germany). The percentage of gap closure was quantitatively measured using Adobe photoshop CS4 software.

Flow Cytometry

Cells were incubated with appropriate dilutions of the following monoclonal antibodies (derived either from BD biosciences or eBioscience) for 30 min at 4°C and analysed on BD Calibur. FITC-labeled antibodies used were anti-CD11c (N418), -CD4 (RM4-5), -NK1.1 (PK136 ebio), -MHC-I (H-2Kb; AF688.5BD),-CD8b (eBIOH35). PE-labeled antibodies were anti-CD8b (eBioH35-17.2), -CD11b (M1/70), -CD80 (16-10A1), -CD86 (GL1), -CD40 (1C10), -MHC-II (M5/114.15.2). APC-labeled antibodies were anti-FoxP3 (FJK-169), anti- F4/80 (BM8). Biotin-labeled plant-derived lectins used were *Maackia Amurensis* (MAA) and *Sambucus Nigra* (SNA) both from Vector Labs, CA, USA, and binding was detected with PE- labeled Streptavidin (Jackson Immunoresearch, UK).

qRT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) combined with a DNase treatment to remove contaminating DNA (Qiagen). cDNA was synthesized using the Reverse Transcription System kit (Promega, WI, USA) following manufacturer's guidelines. Real time PCR reactions were performed using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems), with GAPDH as internal control. Samples were analyzed in triplicate and normalized to GAPDH. Primers were obtained from Invitrogen (Carlsbad, California) and sequences were as follows:

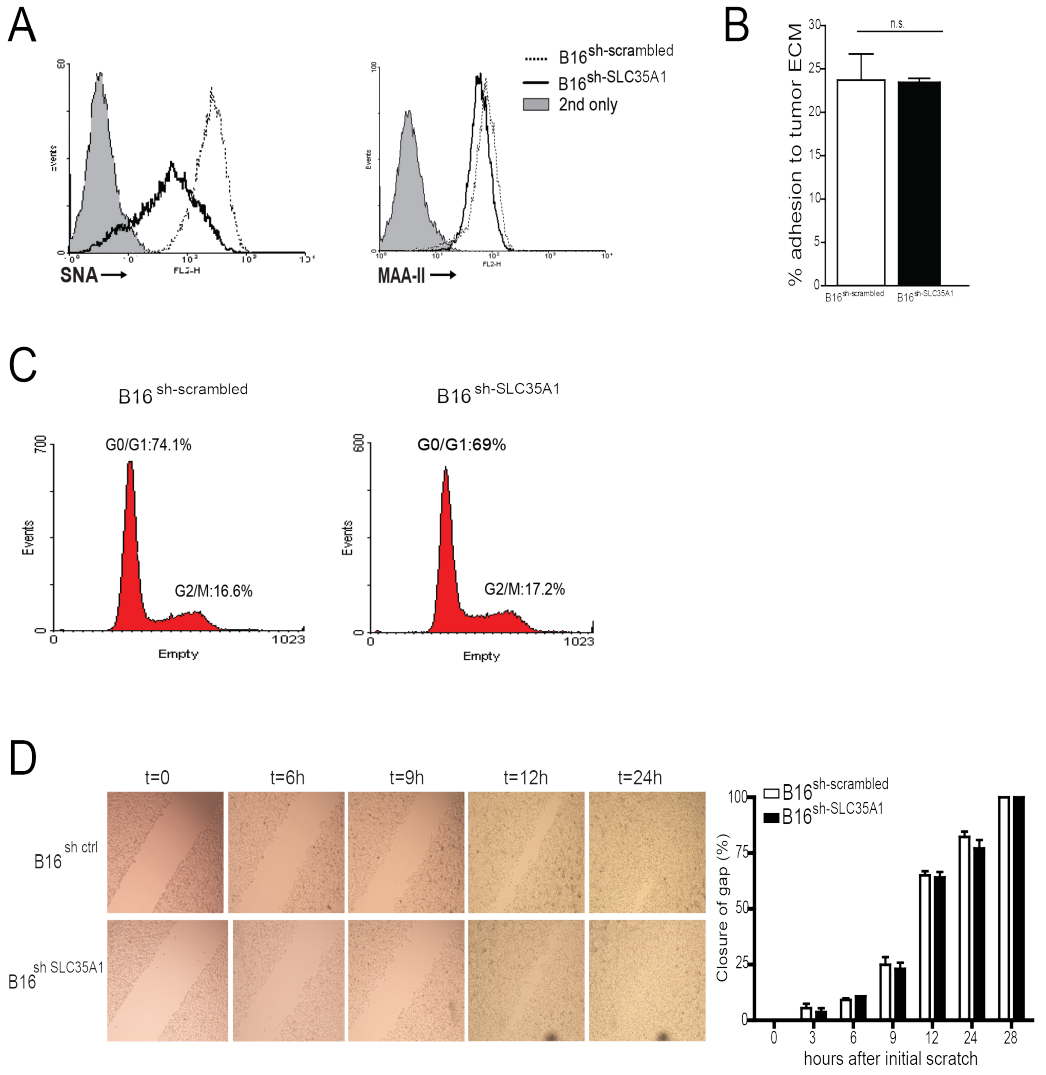

Figure 1. SLC35A1 knockdown in B16-OVA reduces tumor sialylation without altering tumor characteristics in-vitro. A, detection of α2,3- and α2-6-Sias using plant lectins MAA and SNA on $B16^{SLC35A1}$ (black line) and $B16^{scrambled}$ (dashed line) tumors by flow cytometry. Grey filled histograms represent conjugate control. Analysis of sialylation was performed at least three times. B, analysis of tumor cell adhesion to matrigel-coated plates.≠ Results are shown as percentage of cells adhering (mean ± s.e.m). Data are representative of 3 independent experiments. ns., not significant (Student's t test). C, cell cycle analysis of $B16^{SLC35A1}$ and B16scrambled tumors by DNA content. Percentage of cells in G0/G1 interphase and G2/M mitotic phase are indicated. Results are representative of 3 experiments. D, migratory capacity of $B16^{SLC35A1}$ and B16scrambled tumors was addressed via the scratch assay. Left, Bright-field images (200×) of confluent tumor cells showing re-growth following in vitro scratch. Right, quantification of distance between edges of linear scratch at time-points analysed. Data are representative of 3 independent experiments. ns, not significant (Student's t test).

Statistical analysis

Prism software (GraphPad 5.0) was used for statistical analysis. Student's t test was to determine statistical significance. Statistical significance for all the tests, assessed by calculating the P values, was defined as $P<0.05$.

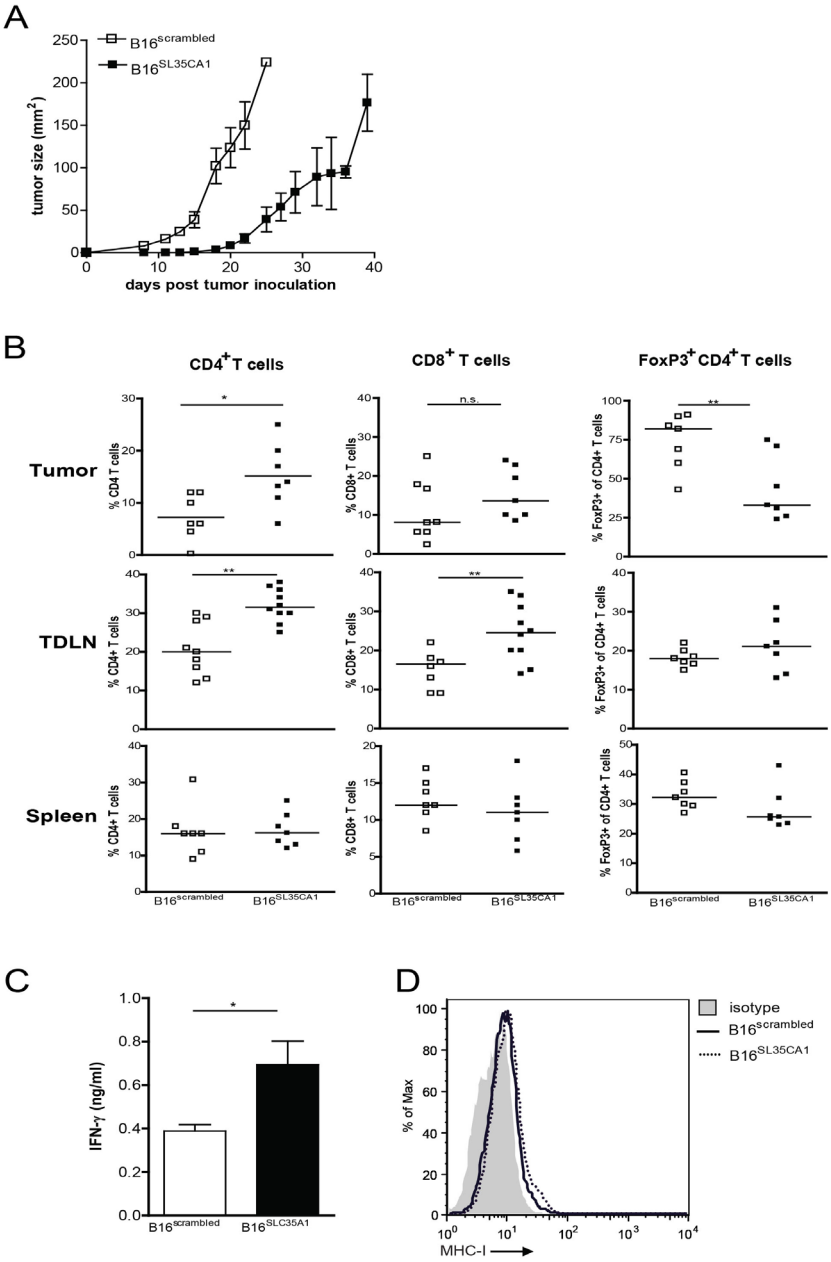

Figure 2. SLC35A1 knockdown delays tumor growth, increases intra-tumoral effector T cells and reduces intra-tumoral Tregs A, tumor growth in $B16^{SLC35A1}$ and $B16^{scrambled}$ tumor-bearing mice (n=7/group). Tumor growth assessed at different days after tumor inoculation is indicated as mm^2 (mean ± s.e.m). B, percentage of total $CD4^+$ and $CD8^+$ T-cells and $CD4^+$ $FoxP3^+$T cells in the tumor, TDLNs and spleen from tumor-bearing mice as detected by flow cytometry at time of sacrifice. Dots represent individual mice (n=7). Bars indicate median of each group. C, IFN-γ levels secreted by TILs from $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors as determined by ELISA. Data are representative of 2 independent experiments. Statistical significance is indicated. ns, not significant (Student's t test). D, Flow cytometric analysis of MHC-I expression on $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors before injection into mice. Plots are representative of two independent measurements.

Results

Reduction of Sias on B16-OVA surface antigens by knock down of *SLC35A1*

To investigate whether Sias on tumors influence host anti-tumor immunity, we reduced the expression of Sias on the hyper-sialylated murine B16-OVA melanoma cells by knocking down solute carrier family 35 member A1 (*SLC35A1*) gene using specific shRNA. *SLC35A1* encodes the CMP-sialic acid transporter, localized in the Golgi membrane and responsible for carrying CMP-Sias into the Golgi to be added to terminal position on glycan chains of nascent glycoconjugates [28]. *SLC35A1* knock down specifically decreased the quantity of α2-6- linked Sias on the surface of B16-OVA compared to B16-OVA that was treated with a non-targeting shRNA (hereafter called $B16^{SLC35A1}$ and $B16^{scrambled}$, respectively) when analysed for binding by the plant lectin SNA, which is specific for α2-6-linked Sias (Fig. 1A). Levels of α2-3-linked Sias were not markedly affected, as demonstrated by equal MAA binding.

Since aberrant sialylation has been correlated with the invasive and metastatic properties of tumors, we next evaluated whether reduction of α2-6-Sias on B16-OVA altered its behavior in-vitro. We started to assess whether reduction of Sias on B16-OVA cells altered their adhesion abilities to extra-cellular matrix (ECM) components. However, no difference between $B16^{SLC35A1}$ and $B16^{scrambled}$ tumor cells in binding to plates coated with tumor-like ECM-components could be observed as indicated by equal percentages of binding by both tumor types (Fig. 1B). We next determined whether their metastatic potential was affected by examining the proliferative and migratory capacity of $B16^{SLC35A1}$ tumors. Cell-cycle analysis revealed that equal percentages of $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors were present in the interphase (i.e. approx 70% of cells) and mitotic phase (i.e. approx 17% of cells) of cell-cycle, indicative of similar proliferative potential (Fig. 1C). The migratory capacity of the tumor cells was determined using the so-called scratch assay. Hereto, a scratch was generated in a tumor cell monolayer and tumor cell migration and closure of the gap was monitored by capturing images at 3h intervals (Fig 1D, left panel). Quantification of these data showed that

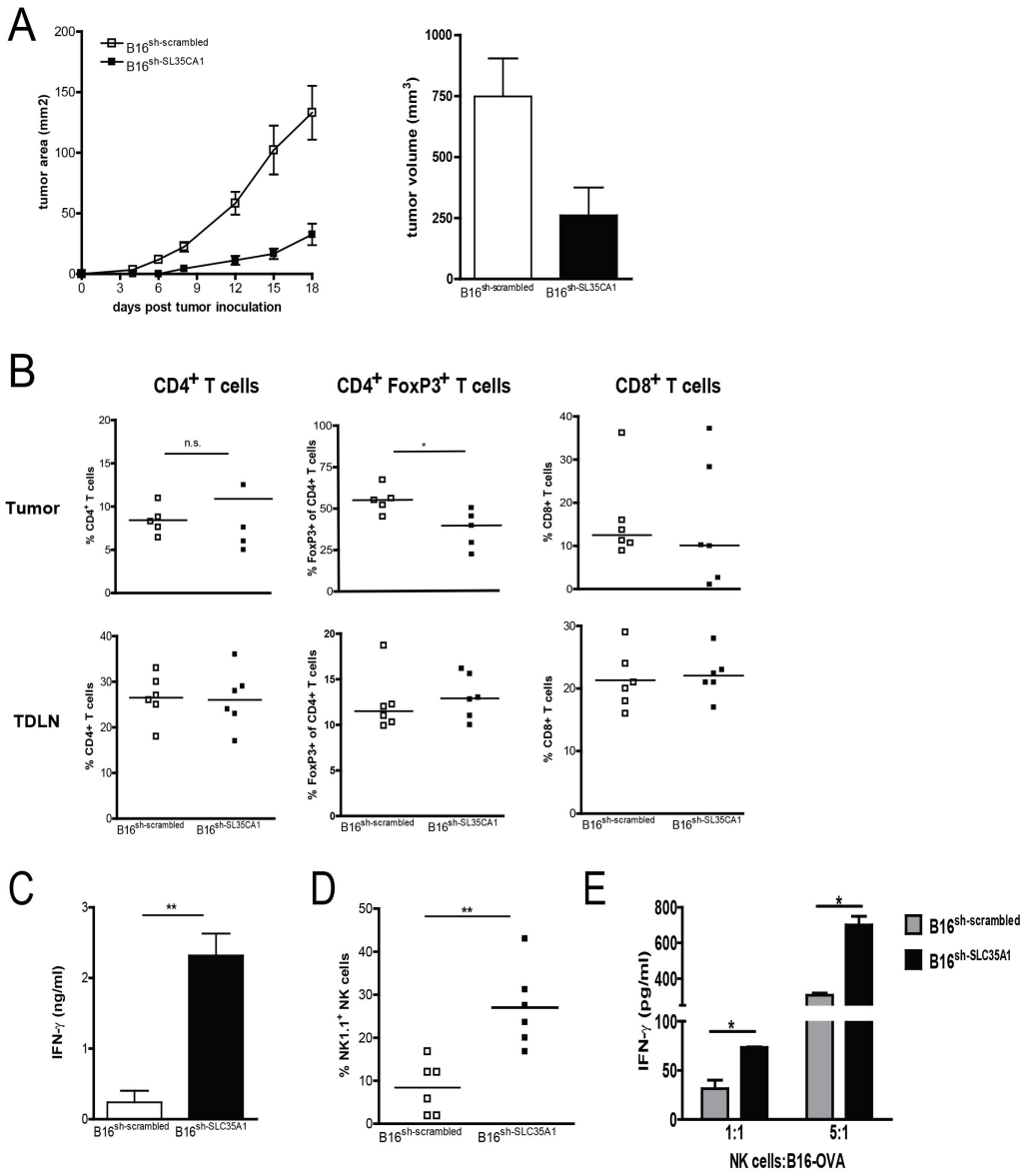

Figure 3. Decreasing sialylation of B16 tumors facilitates rapid increase in intra-tumoral activated NK cells. A, Area (left) and volume (right) of $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors on day 18 after tumor inoculation (n=6 mice per group) calculated in mm^2 and mm^3 (Mean ± s.e.m). Statistical significance is indicated (Student's t test). B, Percentages of total $CD4^+$ T- cells, $CD8^+$ T-cells and of $FoxP3^+$ $CD4^+$ T-cells in the tumor and TDLNs from tumor-bearing mice as detected by flow cytometry. Dots represent individual mice (n=7). Bars indicate median of each group. Statistical significance is indicated (Student's t test). C, Levels of IFN-γ secreted by TILs from $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors upon PMA/ionomycin activation. Data are representative of 2 independent experiments (mean ± s.e.m.). D, percentage of NK cells, distinguished as $NK1.1^+$ $CD3^-$ cells, within $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors (n=6/group) using flow cytometry. Bars indicate median. E, NK cells, enriched from naive splenocytes, were incubated at indicated E:T ratio's from $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors and IFN-γ was determined by ELISA from the supernatant 4h later.

both the B16^{SLC35A1} and B16^{scrambled} tumors migrated at the same rate and were able to close the gap within 28 hours (Fig. 1D). B16^{scrambled} tumors showed comparable characteristics to unmodified WT B16 tumor cells (Fig. S1).

Thus, a reduction of the amounts of α2-6-linked Sias on the surface of B16-OVA melanoma expressing did not alter tumor intrinsic characteristics *in vitro*.

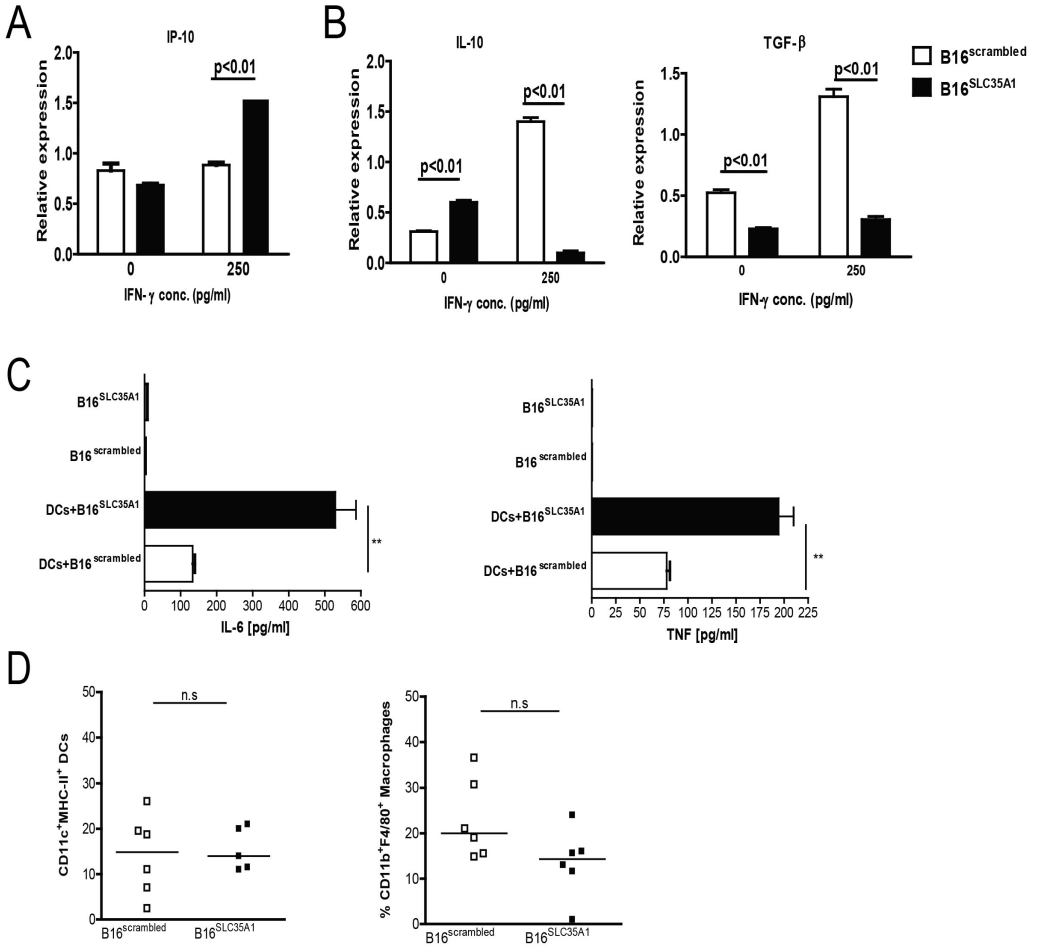

Figure 4. IFN-γ ignites an immunogenic milieu in B16^{SLC35A1} tumors. A+B, IL-10, TGF-β and IP-10 mRNA levels expressed by B16^{SLC35A1} and B16^{scrambled} tumors in the presence or absence of rm-IFN-γ (n=4 independent experiments). C, IL-6 and TNF levels in supernatants of triplicate co-cultures of BMDCs and tumor cells. Supernatant of tumor cells cultured alonewas used as control (n=4). D, percentages of CD11c^{+} MHC-II^{+} DCs and CD11b^{+} F4/80^{+} macrophages within B16^{SLC35A1} and B16^{scrambled} tumors as determined by flow cytometry on day 18 after tumor challenge. Individual mice are shown, n=6/group. Bars indicate the median/group. ns, not significant (Student's t test).

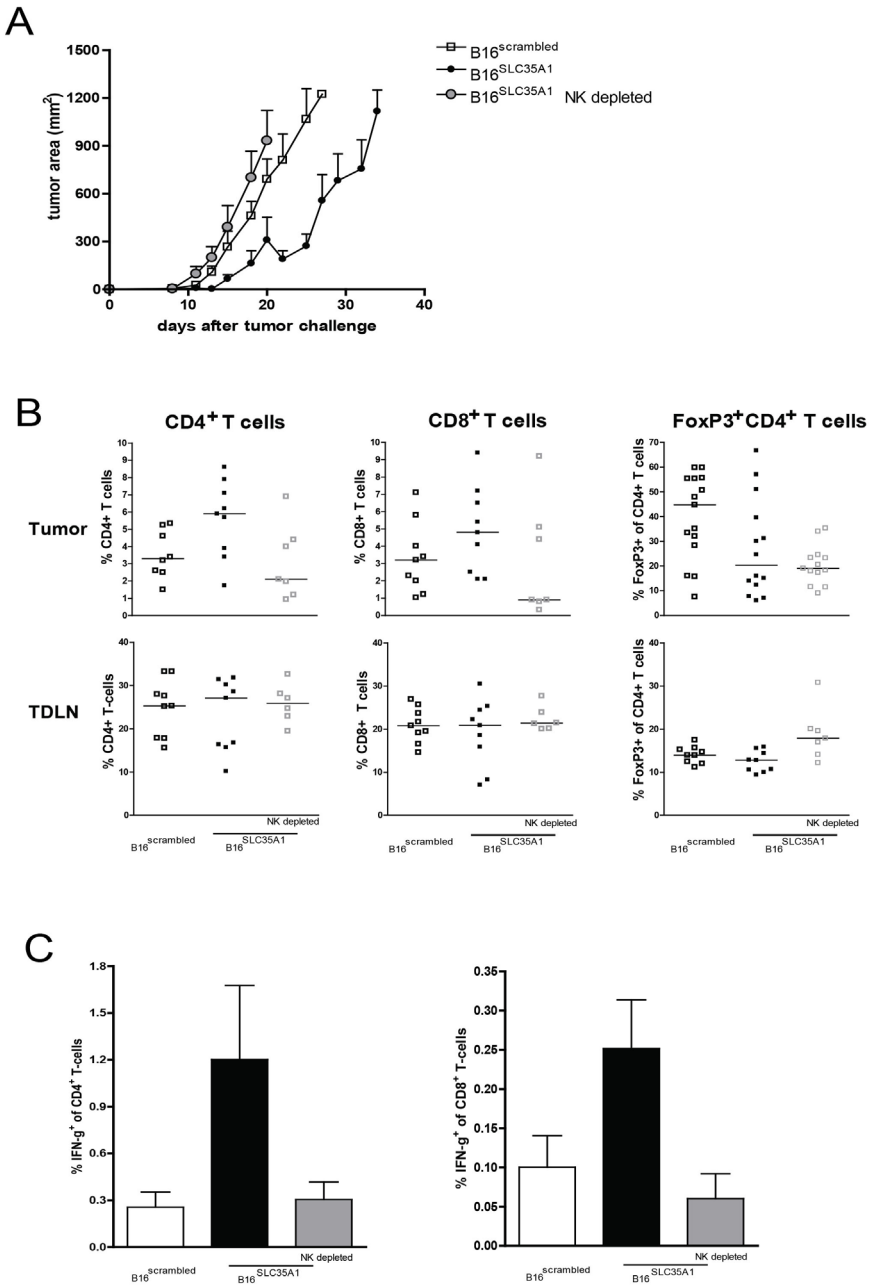

Figure 5. Depletion of NK cells in Sia^{low} tumor bearing mice abolishes induction of anti- tumor immunity. A, NK-depleted mice were challenged i.d. with B16^{SLC35A1} tumor cells; WT mice were challenged with B16^{SLC35A1} or B16^{scrambled} tumor cells. Tumor growth was assessed at different days after tumor inoculation, indicated as mean mm^2 (± s.e.m). Data represent results from two independent experiments with 6-7 mice/group. B, TILs and TDLNs were analysed by flow cytometry to determine the frequency of CD4$^+$ and CD8$^+$ T cells and of FoxP3$^+$ CD4$^+$ T cells. C, IFN-γ production by activated CD8$^+$ and CD4$^+$ T cells in TDLNs was determined by intracellular staining after PMA/ionomycin restimulation ex vivo. Each dot represents one mouse. n=5 mice/group. * P< 0.05, ** P<0.01, *** P<0.001. Graphs shown are representative of two independent experiments.

Reducing Sias on tumors switches the T cell response from tolerogenic into immunogenic

The generation of B16-OVA with reduced expression of Sias enabled us to study the implication of tumor sialylation on the host anti-tumor immunity. Either $B16^{SLC35A1}$ or $B16^{scrambled}$ tumors were i.d. injected into the flank of immunocompetent C57BL/6 mice. $B16^{scrambled}$ tumors were already visible 7 days after inoculation and grew substantially faster and larger than $B16^{SLC35A1}$ tumors, which started to be detectable approximately 15 days after tumor implantation and remained much smaller in size for a prolonged period (Fig. 2A).

We hypothesized that the reduced growth of $B16^{SLC35A1}$ tumors arose from changes in the host's anti-tumor immune response as their proliferative and migratory capacities were equal (Fig 1B-D). We therefore examined the composition of tumor-infiltrating lymphocytes (TILs), tumor-draining lymph nodes (TDLNs) and spleen by flow cytometry at time of sacrifice (i.e. day 20 for $B16^{scrambled}$ and day 40 for $B16^{SLC35A1}$). In particular, a higher percentage of $CD4^+$ T-cells were detected within the TILs and TDLNs of mice bearing $B16^{SLC35A1}$ tumors (Fig. 2B). Likewise, we found increased $CD8^+$ T cell proportions in the TILs and TDLNs (Fig. 2B). Notably, we observed a reduced fraction of $CD4^+$ $FoxP3^+$ T cells in the $B16^{SLC35A1}$ microenvironment (Fig. 2B). Together with the strong elevated levels of the effector cytokine IFN-γ secreted by $B16^{SLC35A1}$-infiltrating lymphocytes upon *ex vivo* re-stimulation (Fig. 2C), this suggests that the $CD4^+$ and $CD8^+$ T cells in the $B16^{SLC35A1}$ tumors are effector rather than tolerogenic T cells. In contrast to our observation on TILs, no reduction in the proportion of $CD4^+$ Tregs was measured in TDLNs of $B16^{SLC35A1}$ tumors (Fig. 2B, lower panels). Furthermore, no significant differences in T-cell numbers and phenotype were observed in the spleens of tumor-bearing mice, indicating that anti-tumor immunity is confined to the tumor-environment (Fig. 2B). As expression levels of MHC class I molecules were comparably low between the two tumor types (Fig. 2D), it can be ruled out that a difference in MHC-I levels on $B16^{SLC35A1}$ tumors ensures better recognition by tumor-specific $CD8^+$ T cells than $B16^{scrambled}$ tumors. Together, these findings clearly show that a reduction of Sias on tumor surfaces evokes a switch in the type of T cells residing in the tumor and TDLNs: instead of regulatory T cells effector T cells are

present, which control tumor growth.

Sias on tumors dampen activity of NK cells and DCs

To analyze whether the delayed growth of $B16^{SLC35A1}$ tumors *in vivo* was paralleled by variations in innate cell subsets, which are predominantly involved in the first phases of cancer immunoediting, mice were sacrificed 18 days after tumor implantation, coinciding with early phases of $B16^{SLC35A1}$ tumor development (Fig. 3A, left panel). As expected, $B16^{SLC35A1}$ tumors were significantly smaller in size than $B16^{scrambled}$ tumors (Fig. 3A, right panel). Analysis of the T cells within $B16^{SLC35A1}$ tumors 18 days after tumor injection revealed the same increase in total $CD4^+$ T cells found at a later stage (i.e. 40 days after implantation; Fig. 3B + 2C). Also, a markedly lower $CD4^+$ $FoxP3^+$ T cell fraction was present than in the $B16^{scrambled}$ tumors (Fig. 3B). No significant changes in tumor-infiltrating $CD8^+$ T cells were detected at this early stage of tumor development (Fig. 3B). Similar as before the reduced frequency of $CD4^+$ Tregs in $B16^{SLC35A1}$ tumors was concomitant with the increased presence of IFN-γ production by infiltrating lymphocytes, indicating a shift from T cell tolerance towards T cell immunity that sets stage early after tumor implantation (Fig. 3C). Interestingly, $B16^{SLC35A1}$ tumors contained 25% of $NK1.1^+CD3^-$ NK cells, while in $B16^{Scrambled}$ tumors only 10% was detected (Fig. 3D). NK cells are amongst the first responders against tumor development, where they actively kill the transformed cells by secreting IFN-γ. This function may be inhibited in hypersialylated tumors [29]. Since we observed such high frequencies of NK cells in $B16^{SLC35A1}$ tumors we tested whether co- culturing NK cells *in vitro* with $B16^{SLC35A1}$ or $B16^{scrambled}$ tumors would alter the NK cell activity as measured by IFN-γ production. The IFN-γ levels in the supernatants clearly demonstrate that a reduction in Sias increased the IFN-γ production by NK cells, at each E:T ratio tested (Fig. 3E).

In addition to its cytolytic effects, IFN-γ can also induce tumor cells to produce chemokines that attract immune effector cells, such as CXCL10 (IP-10) [30, 31]. To examine this, $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors were cultured in the presence or absence of IFN-γ, and IP- 10 mRNA levels were established by qRT-PCR. The

presence of IFN-γ clearly increased the levels of IP-10 mRNA in $B16^{SLC35A1}$ but not in $B16^{scrambled}$ (Fig. 4A).

Of note, both tumor types expressed equal levels of IFN-γ receptor mRNA (Fig. S2). Furthermore, we found that IFN-γ strongly reduced mRNA expression levels of the anti-inflammatory cytokines IL-10 and TGF-β in the $B16^{SLC35A1}$, thus making the tumor micro-milieu of the Sia^{low} tumor less tolerogenic (Fig. 4B). Although no significant changes in numbers of DCs, discerned by $CD11^+$ $MHC\text{-}II^+$ co-expression, in $B16^{SLC35A1}$ tumors were observed (Fig. 4D), these DCs produced elevated levels of the pro-inflammatory cytokines IL-6 and TNFα when co-cultured with $B16^{SLC35A1}$ tumors *in vitro* (Fig. 4C). Likewise, no differences in the numbers of $CD11b^+$ $F4/80^+$ macrophages (19 vs 12%, respectively) were detected between mice with $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors (Fig 4D). Thus, hyper-sialylated tumor cells paralyze the inflammatory program of DCs and macrophages and attenuate NK cell activation and herewith maintain a tolerogenic environment.

Presence of NK cells is required to evoke the switch in T-cell response in Sia^{low} tumors

The presence of higher numbers of IFN-γ-secreting NK cells in $B16^{SLC35A1}$ tumors (Fig. 3D) as well as the immunogenic effect of IFN-γ on the tumor milieu, suggests that NK cells play a relevant role in the control of $B16^{SLC35A1}$ growth. To test this, mice challenged with $B16^{SLC35A1}$ tumors were depleted of NK cells by treatment with anti-NK1.1 antibodies and tumor growth as well as immune parameters was assessed. Near complete NK cell depletion was confirmed by flow cytometry (Fig. S3). NK depletion drove $B16^{SLC35A1}$ to grow at a similar rate as $B16^{scrambled}$ (Fig. 5A). As observed previously, $B16^{SLC35A1}$ tumors grew slower and were smaller in size when implanted in WT NK-competent mice, leading to survival of mice 35 days after tumor transplantation (Fig 5A+2A). Focusing on the immune composition, we observed that the increased infiltration of $CD4^+$ and $CD8^+$ T cell into $B16^{SLC35A1}$ tumors was absent when NK cells were depleted (Fig. 5B). This was mirrored by the absence of effector T cells in these mice: IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were only detected in $B16^{SLC35A1}$

tumors (Fig. 5C). Moreover, the proportion of $CD4^+$ $FoxP3^+$ T cells was significantly reduced in $B16^{SLC35A1}$ tumors. Depletion of NK cells did not alter the proportion of $CD4^+$ $FoxP3^+$ T cells within the tumor, however Treg numbers were significantly higher in the TDLNs of NK cell depleted $B16^{SLC35A1}$ tumor bearing mice (Fig. 5B). In summary, these data unveil a clear and versatile role for the levels of Sias on tumors regulating NK cell mediated initiation of potent anti-tumor immunity.

Discussion

Here we show that reducing Sias on the surface of melanoma cells evokes the generation of large numbers of effector $CD4^+$ and $CD8^+$ T-cells that infiltrate the tumor area and simultaneously dampens the intra-tumoral presence of Tregs, together curtailing tumor growth. Furthermore, we provide evidence that this switch in T-cell response from tolerogenic to immunogenic in Sia^{low} melanomas is ignited by NK activation as their depletion abolishes effector T-cell induction.

In this study we reduced the amounts of Sias on the murine B16-OVA melanoma surface to assess the impact of Sia alterations on the anti-tumor immune response. SLC35A1 knock down significantly reduced the presence of α2-6-linked Sias on the B16-OVA surface, while the levels of α2-3-linked-Sias were only slightly reduced. Besides melanoma, also other invasive and metastatic tumors (e.g. colon and breast carcinoma) present an aberrant expression of α2-6-Sias mainly because of the overexpression of ST6Gal1 sialyltransferase which adds terminal Sia moieties on N-linked glycans [12]. It has been shown that enhanced expression of α2-6-Sias on tumors promotes cell detachment from the tumor mass as well as their invasiveness by facilitating interactions with matrix proteins [32, 33].

We observed that in-vivo $B16^{SLC35A1}$ tumors grew significantly slower and remained much smaller in size than $B16^{scrambled}$ tumors for a long period, resulting in better survival of mice bearing $B16^{SLC35A1}$ tumors. Since *in vitro* intrinsic properties of B16-OVA such as migratory and adhesion abilities were not affected by the reduction of α2-6-Sias, the delayed growth of $B16^{SLC35A1}$ tumors *in vivo* is the outcome of improved immune control. This is also underlined by our findings that $CD4^+$ and $CD8^+$ T-cell numbers were increased in the tumor and TDLNs as well as their ability to produce IFN-γ. At the same time, frequencies of tumor- associated Tregs were strongly diminished in the $B16^{SLC35A1}$ tumors. Thus, reducing Sia on the tumor surface switches the function of tumor-specific T-cells from tolerogenic to effector.

Analysis of the immune composition in the early stage of tumor growth revealed increased frequencies of NK cells in $B16^{SLC35A1}$ tumors compared to $B16^{scrambled}$ tumors. Moreover, NK cells engaging $B16^{SLC35A1}$ tumor cells secreted elevated

amounts of IFN-γ compared to those engaging $B16^{scrambled}$ cells. A similar enhanced IFN-γ-secretion by NK cells was reported in a study in which sialylation of the chemically induced fibrosarcomas was reduced via sialidase treatment [34].

NK cells express Siglec receptors such as Siglec-E and Siglec-G [35, 36], which bear specificity for α2-6-linked sialylated branches [16]. Thus, it is possible that the reduction of α2-6-Sias on B16-OVA diminished Siglec-mediated inhibition of NK cell functions. Indeed, a recent study showed that the susceptibility of tumor cells to NK cytotoxicity could be blocked by Siglec-blocking antibodies [37]. The absence of this inhibitory interaction paves the way for the NK activating receptor NKG2D with its ligands on tumors, enhancing IFN-γ secretion and killing capacity of NK cells [34]. The augmented numbers of $NK1.1^+$ NK cells during early stages of tumor development could be a result of specific chemokines secreted by Sia^{low} tumor that attract NK cells to the tumor site. Since IFN-γ has been found to mediate recruitment of NK cells to sites of infection [38], the high numbers of NK cells found in Sia^{low} tumors are likely the result of initial IFN-γ producing NK cells that attracts other NK cells to the Sia^{low} tumor milieu.

It has been reported that NK cell-produced IFN-γ contributes to the DC-mediated $CD4^+$ and $CD8^+$ T cell activation [39, 40]. The absence of effector $CD4^+$ and $CD8^+$ T cells in $B16^{SLC35A1}$ tumors and TDLNs when NK cells were depleted elucidates then that the IFN-γ released by activated NK cells likely also functions to activate DCs. Indeed, we showed that DCs in $B16^{SLC35A1}$ but not in $B16^{scrambled}$ tumors display an immunogenic phenotype reflected by the production of pro-inflammatory cytokines. This activated phenotype of DCs is instrumental in the subsequent induction of large amounts of effector T-cells. Additionally, the released IFN-γ makes the tumor micro-milieu susceptible for immune attack as we showed that IFN-γ reduced production of anti-inflammatory cytokines by tumor cells.

Next to the IFN-γ released by activated NK cells, this may also be the result of diminished siglec triggering on DCs. Similar to NK cells, DCs also express siglecs, and predominantly Siglec-E. It has been described that binding of sialylated pathogens to siglecs on DCs dampens secretion of pro-inflammatory cytokines [19]. Moreover, DC binding of sia structures on C. jejuni modulates the signals DCs provide to naive T-cells and that mediate T cell activation and polarization. It is likely that enhanced tumor sialylation adds to the generation of tolerogenic

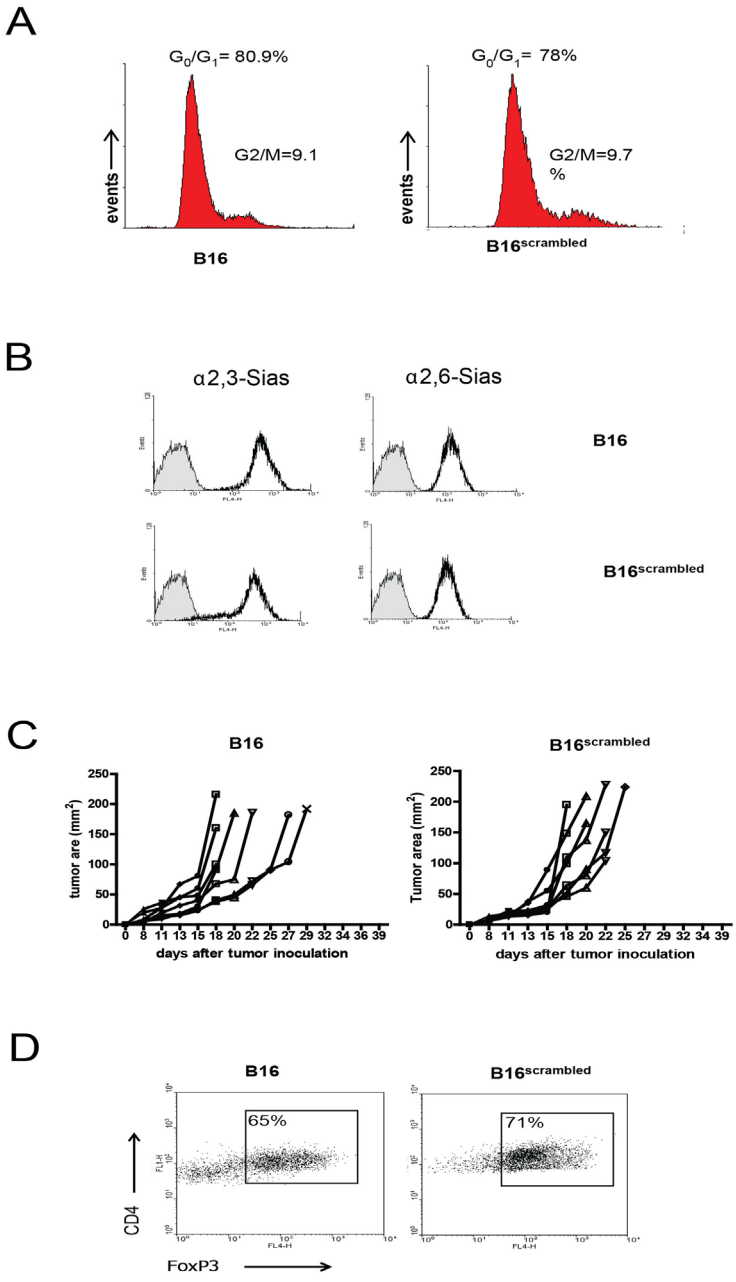

Fig S1. B16^{scrambled} behaves similar to WT B16-OVA in-vitro and in-vivo. A, cell cycle analysis of B16 and B16^{scrambled} tumors by DNA content. Percentage of cells in G0/G1 interphase and G2/M mitotic phase are indicated. Results are representative of 3 experiments. B, detection of α2,3- and α2,6-Sias using MAA and SNA on B16 and B16^{scrambled} tumors (black lines) by flow cytometry. Gray filled histograms represent conjugate control. Analysis of sialylation was performed at least three times. C, tumor growth in B16 and B16^{scrambled} tumors-bearing mice (n=7). Tumor growth assessed at different days after tumor inoculation is indicated as mm^2 (mean ± s.e.m). D, Analysis of intra-tumoral CD4$^+$ FoxP3$^+$ T cells in B16 and B16^{scrambled} tumors by flow cytometry. Percentage of CD4$^+$ FoxP3$^+$ T cells is shown.

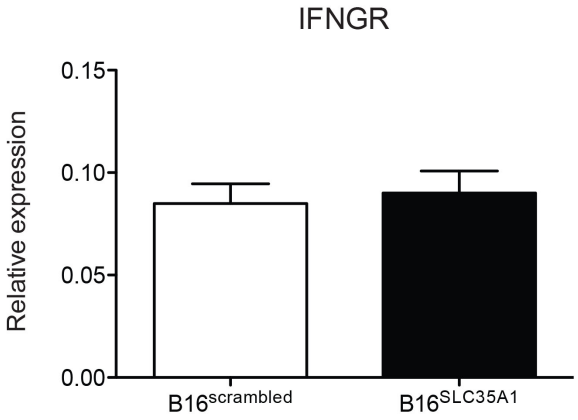

Fig S2. B16^{SLC35A1} and B16^{scrambled} have equal expression level of IFN-γ receptor mRNA. Expression of IFN-γ receptor (IFNGR) in B16^{SLC35A1} and B16^{scrambled} tumors as determined by qRT-PCR. Gene expression was normalized and presented as relative expression to GAPDH. Values shown are the mean ± s.e.m of two experiments.

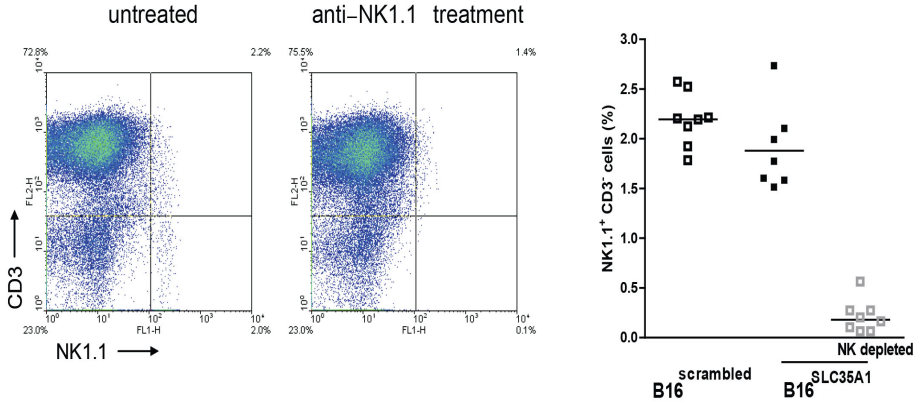

Fig S3. Absence of NK cells in NK-depleted B16^{scrambled}-bearing mice. Detection of NK cells in spleens from untreated and NK-depleted tumor-bearing mice (n=7/8 per group) by flow cytometry. Spleens were stained for NK1.1 and CD3. Graph shows percentage of NK1.1^{+} CD3^{-} cells (median).

above: Gustav Klimt; Death and life (1910/15). Leopold Museum, Vienna.

Chapter 3

Sialic acid–modified antigens impose tolerance via inhibition of T cell proliferation and *de novo* induction of regulatory T cells

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Abstract

Sialic acids (Sias), terminal carbohydrate structures on host cell proteins and certain pathogens, interact with inhibitory Siglec receptors, suggesting a regulatory role in homeostasis or pathogen-mediated immune modulation. Using two independent antigens we demonstrate that modification with Sias (Sia-antigen) drastically alters dendritic cell (DC) and T cell responses. DC uptake of Sia-antigen strongly promoted de novo $CD4^+$ regulatory T cell (Treg) induction while inhibiting IFN-γ-producing T cell differentiation. In vivo, injection of Sia-antigen increased Treg numbers, and dampened effector T cell expansion and IFN-γ production. Inhibition of effector T cells required lower amounts of Sia-antigen than Treg induction. Sia-antigen imposed dual tolerogenic features on DCs upon Siglec-E-mediated internalization, even when DC stimuli were applied simultaneously.
In conclusion, sialylation alters antigen immunogenicity and provides a novel way to induce antigen specific immune tolerance.

Introduction

Dendritic cells (DCs) reside in tissues where they continuously sample the environment for dangerous cues. When they encounter a pathogen, DCs undergo maturation which provides the DCs with the crucial signals necessary for proper $CD4^+$ T cell activation and differentiation (Janeway, Jr. and Medzhitov, 2002). Depending on the co-stimulatory and cytokine signals provided by DCs, naive $CD4^+$ T helper (Th) cells differentiate into Th1, Th2, or Th17 cells (Zhu et al., 2010).

Campylobacter jejuni and *Neisseria meningitidis* have been shown to negatively affect human antigen presenting cell (APC) function and consequently subvert immune responses. A common feature of these pathogens is high level expression of sialic acids (Sias) on their surface (Bax et al., 2011; Guerry et al., 2002; Harvey et al., 2001; Jales et al., 2011; Severi et al., 2007; van Vliet et al., 2008; Varki and Gagneux, 2012). Sias are the most prevalent terminal monosaccharide present on the surface of mammalian cells. Sias can be α2-3-, α2-6-, or α2-8-linked to the underlying glycan, and are often found at the outer ends of surface-exposed oligosaccharide chains attached to proteins and lipids (Varki and Schauer, 2009). Given their terminal position, Sias serve as ligands for siglec receptors, a large family of sialic acid-binding Ig-like lectins (Crocker et al., 2007; Varki and Schauer, 2009). Siglecs display a restricted pattern of expression and are predominantly found on innate immune cells, such as DCs, macrophages and NK cells, as well as B cells. The CD33/Siglec-3-related siglec (CD33rSiglecs) subgroup of siglecs possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their intracellular portion, which can counteract the activating signals triggered by receptors containing immunoreceptor tyrosine-based activatory motifs (ITAMs) (Crocker et al., 2007). This suggests that engagement of hCD33rSiglecs on innate cells by Sia-antigens may result in suppressive effects on the innate immune response. Indeed, increased sialylation of pathogen-derived Sia-bearing gangliosides prevented the activation of DCs, as indicated by a reduction in co-stimulatory molecule upregulation and secretion of IL-6 and IL-12, which together resulted in reduced Th1 generation (Guerry et al., 2002; Jales et al., 2011). Furthermore, the microbicidal abilities of neutrophils are suppressed upon binding of group B

Streptococcus to Siglec-9 (Carlin et al., 2007; Carlin et al., 2009). In addition, *Campylobacter jejuni* and *Neisseria meningitidis* are capable of mimicking host structures by decorating their lipo-oligo/polysaccharides with Sias. Binding of these sialylated pathogenic structures to siglecs on DCs inhibits the production of pro-inflammatory cytokines such as IL-12 by DCs (Bax et al., 2011; Erdmann et al., 2009; Steeghs et al., 2006).

Although effects of Sia-bearing antigens on antigen-presenting cell function have been demonstrated, the effects of Sia-bearing antigens on T cell responses are poorly studied. We previously showed that the linkage of Sias on Campylobacter jejuni lipooligosaccharides (LOS) regulates DC-mediated human $CD4^+$ T cell skewing (Bax et al., 2011). In vitro, $CD4^+$ T cells were polarized towards the Th1 lineage when DCs were targeted with α2-8-sialylated LOS, whereas T cells were committed to the Th2 lineage when α2-3-sialylated LOS were used. Notably, increased frequencies of $CD4^+$ $FoxP3^+$ regulatory T cells (Tregs) have been associated with *Neisseria gonhorroeae* and also Herpes simplex viral infections (Imarai et al., 2008; Nandakumar et al., 2009). Both pathogens are hyper-sialylated; however, whether Sias expressed by these pathogens are involved in regulation of the number and/or induction of Tregs by modulating DC function is unknown. Therefore, we investigated whether uptake of sialylated antigens by DCs affects their $CD4^+$ T cell polarizing capacity. As most of the immune-inhibitory effects induced by sialylated pathogens can not be attributed to specific Sias and/or siglecs, we designed well defined synthetic antigen conjugates composed of either the model antigen ovalbumin or a peptide derived from myelin oligodendrocyte glycoprotein (MOG_{35-55}) to which α2-3- or α2-6- linked Sias were chemically coupled. Our data reveal that internalization of Sia-antigen by DCs endows them with the ability to promote the differentiation of naive $CD4^+$ T cells into Tregs at the expense of functional effector T cells both in vitro and *in vivo*. We provide evidence that this feature is imposed on DCs upon Siglec-E-mediated antigen uptake and is even effective under inflammatory conditions.

Materials and Methods

C57BL/6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands) and used at 8-12 weeks of age. OT-II, DO11.10, DO11.10 x Rag2$^{-/-}$ and 2D2 TCR transgenic mice were bred and housed in the animal facilities of VUmc, Erasmus MC, and the TWINCORE Institute under specific pathogen-free conditions. All experiments were approved by the Animal Experiments Committee of the Erasmus MC and VUmc and performed in accordance with national and international guidelines and regulations.

Cells

Splenic DCs. Spleens isolated from C57BL/6 mice were incubated in HE medium (RPMI1640 media containing 10% FCS, 10 mM EDTA, 20 mM HEPES, 50 U/ml penicillin, 50 µg/ml streptomycin) supplemented with 1 WU/ml Liberase-TL (Roche Diagnostics GmbH, Manheim, Germany) and 50 µg/ml DNase I (Roche) for 30 min at 37°C or until digested. Red blood cells were lysed using ACK lysis buffer and undigested material was removed by filtration. DCs were purified by positive selection using anti-CD11c MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The resulting CD11c^{+} MHC-II^{+} populations were typically 94% pure as assessed by flow cytometry.

Bone marrow-derived DCs. BMDCs were cultured as described by Lutz et al. (Lutz et al., 1999) with minor modifications. Briefly, the femur and tibia of C57BL/6, Siglec-E$^{-/-}$, or mSIGNR1$^{-/-}$ mice were removed and the marrow was flushed using Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Carlsbad, CA, USA). The resulting marrow suspensions were passed through 100 µm gauze to obtain single cell suspensions. After washing, 2 x 10^{6} cells were seeded into 100 mm dishes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) in 10 ml IMDM supplemented with 10% FCS and containing 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (BioWhittaker, Walkersville, MD, USA), 50 µM β-mercaptoethanol (Merck, Damstadt, Germany) and 30 ng/ml recombinant murine GM-CSF (rmGM-CSF). On day 2,

10 ml medium containing 30 ng/ml rmGM-CSF was added. On day 5, another 30 ng/ml rmGM-CSF was added to each plate. From day 7 onwards, the non-adherent DCs were harvested and used for the subsequent experiments.

Naive $CD4^+$ T cells. OVA-specific $CD4^+$ T cells were isolated from spleen and lymph node (LN) cell suspensions obtained from OT-II or DO11.10 mice. The LN and spleens were homogenized and naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ T cells were purified using the Dynal mouse $CD4^+$ $CD62L^+$ T cell isolation kit II mouse (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting naive $CD4^+$ $CD62L^+$ $CD25^-$ T cell populations were typically 90% pure as assessed by flow cytometry.

Antibodies

The FITC-labeled antibodies used were anti-CD11c (N418), anti-PDL2 (122), anti-CD25 (PC61.5); the PE-labeled antibodies were anti-CD8b (H35-17.2), anti-CD4 (GK1.5), anti- CD80 (16-10A1), anti-CD86 (GL1), anti-CD40 (1C10), anti-MHC class II (MS/114.15.2), anti-PDL1 (MIH5), anti-Vβ5 (MR9-4); the APC-labeled antibodies were anti-CD62L (MEL- 14), anti-FoxP3 (FJK-169), and anti-IFN-γ (XMG1.2). Biotin-labeled anti-Vα2 TCR (B20.1) was detected using Alexa Fluor 488-labeled streptavidin (Jackson Immunoresearch, WestGrove, PA, USA). Anti-CD86, -MHC-II, -Vα2, -Vβ5, -CD62L and –IFN-γ antibodies were purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA), all other antibodies were obtained from eBiosciences (eBiosciences, San Diego, CA, USA).

Modification of antigens with sialylated glycans

To obtain Sia-OVA and Sia-MOG, maleimide-activated 6'-sialyl-N-acetyllactosamine (SLN306; Neu5Acα2-6Galβ1-4Glc; DEXTRA Labs, UK) and 3'-sialyl-N-acetyllactosamine (SLN302; Neu5Acα2-3Galβ1-4Glc) were conjugated to thio-activated OVA (Calbiochem, Germany) and to MOG_{35-55} peptide with an added cysteine to the N-terminus, through a thiol-ene reaction. MOG_{35-55} peptide was produced by solid phase peptide synthesis using Fmoc chemistry with a Symphony peptide synthesizer (Protein Technologies Inc., USA).

The glycans were activated with the bi-functional cross linker 4-N-maleimidophenyl butyric acid hydrazide (MPBH, Pierce, USA) and OVA was activated with the linker N-succinimidyl S-acetylthioacetate (SATA; Pierce, USA).

The hydrazide moiety of MPBH was covalently linked to the reducing end of the carbohydrate via reductive amination at a 3:1 molar ratio, OVA reacted with SATA via the amino groups on its surface at a 6:1 molar ratio, and the final reaction of OVA-SATA with the derivatized carbohydrate was performed at a molar ratio of 1:10. The final reaction of MOG_{35-55} with the derivatized carbohydrates was performed at a molar ratio of 1:1.5.

Briefly, SATA, dissolved in DMSO, was added to a filtered solution of OVA in phosphate buffer (pH = 8.2; filtered to 200 nm). After vigourous stirring, the resulting OVA-$SATA^{Ac}$ solution was purified using a PD10 column (GE Healthcare, The Netherlands) and diluted with 10% v/v of 0.5 M NH2OH*HCl pH 7.2 solution. After another 40 min of vigorous stirring and a second purification using a PD10 column, OVA-$SATA^{SH}$ was ready for coupling with the MPBH-glycans. A mixture of MPBH (3 eq.), 3'-sialyl-N-acetyllactosamine (or 6'-sialyl-N-acetyllactosamine) (1 eq.) and picolineborane (10 eq., Sigma-Aldrich, Germany) dissolved in DMSO/AcOH (8:2) was incubated for 2 h at 65°C, cooled to RT, 1.4 ml of ice-cold isopropanol (anhydrous; Sigma-Aldrich) was added, and then incubated at – 20°C for 1 h. Subsequently, the precipitated MPBH-carbohydrates were pelleted, washed twice with cold isopropanol, and dissolved in 50 µl of PBS. The derivatization was confirmed by electrospray ionization mass spectrometry. The obtained MPBH-3'sialyl-N- acetyllactosamine and MPBH-6'-sialyl-N-acetyllactosamine were used immediately for coupling to OVA-$SATA^{SH}$ and to MOG_{35-55} peptide. Conjugation of OVA-$SATA^{SH}$ and MOG_{35-55} to the activated glycans was performed o/n at 4°C and the neo-glycoconjugates were purified by size exclusion chromatography. The concentrations of OVA and MOG were determined using the bicinchoninic acid assay (Pierce, USA).

The presence of α2,6- or α2,3-linked-Sias on the antigen was confirmed by an ELISA (Fig. S1). In brief, 10 µg/ml of Sia-antigen was coated directly onto ELISA plates (Nunc Maxisorb; Nunc, Roskilde, Denmark), followed by incubation with the biotinylated plant lectins from Sambucus Nigra or Maackia Amurensis (Vector Labs, Burlingham, CA, USA) and peroxidase-labeled streptavidin

(Sigma-Aldrich). To detect Sia-OVA, OVA-specific monoclonal antibodies (clone A6075; 10 µg/ml, Sigma-Aldrich) were used followed by peroxidase-labeled goat-anti-mouse antibodies.

T helper differentiation and testing of suppressive capacity

Splenic DCs or BMDCs (1 x 10^4) were pulsed with 30 µg/ml Sia-OVA or native OVA for 4 h in round bottom 96-well plates, then 5 x 10^4 purified naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ OT-II T cells were added to each well. After 2 days, 10 U/ml recombinant mouse IL-2 (Invitrogen, Bleijswijk, The Netherlands) was added. T cell polarization was analyzed on day 6. Briefly, the cells were re-stimulated with 30 µg/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin in the presence of 5 µg/ml Brefeldin A (Sigma), incubated for 5 h, and the expression of FoxP3 and IFN-γ were analysed by flow cytometry. To determine whether Sia-OVA-loaded DCs induce Treg differentiation via a soluble factor, the cells were cultured in 0.4 µM pore size Transwell plates (Millipore, Billercia, MA, USA). OVA-pulsed DCs and purified naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ OT-II were co-cultured in the lower chamber, while Sia-OVA-pulsed DCs were plated into the upper chamber.

To determine whether the tolerogenic effects of Sia-OVA-pulsed DCs occur in a cell contact- dependent manner, Sia-OVA-DCs were co-cultured with OVA-DCs and naive OT-II T cells. Sia-OVA–pulsed DCs and naive OT-II T cells were co-cultured as a control. After 2 days, 10 U/ml recombinant mouse IL-2 was added to the lower chamber, and the T cells were analysed for the expression of IFN-γ and FoxP3 on day 7 as described above.

Additionally, Sia-OVA-DC-primed T cells were assayed for suppressive capacity. Briefly, T cells were added to co-cultures of 1 x 10^4 OVA-loaded DCs and CFSE-labeled responder OT- II T cells. Four days later, proliferation of the CFSE-labeled responder T cells was analysed by flow cytometry.

MHC class II-restricted antigen-presentation

DCs (1 x 10^4) were pulsed with the indicated amounts of antigen, and then co-cultured with 5 x 10^4 purified $CD4^+$ OT-II T cells in 96-well round bottom plates.

After 48 h, [^{3}H]thymidine (1 µCi/well; Amersham Biosciences, Piscataway, NJ, USA) was added, then the cells were incubated for 16 h, harvested onto filter paper and [^{3}H]thymidine incorporation was assessed using a Wallac microbeta counter (Perkin-Elmer, Waltham, MA, USA).

Cytonalysis

Cytokine production by T cells and DCs was assessed by cytometric bead arrays using the CBA Th1/2/17 kit and mouse inflammation kit, respectively (BD Biosciences) or by ELISA using specific antibody pairs for IL-10 (eBiosciences) following the manufacturers' instructions.

In vivo treatment with Sia-OVA

For adoptive transfer of antigen-loaded DCs, the DCs were pulsed overnight with 200 µg/ml OVA or Sia-OVA, and 24 h later 3 x 10^5 antigen-pulsed DCs were injected intravenously into each recipient mouse. One week later, the mice were immunized s.c. with a mixture of 100 µg OVA and 50 µg CpG.

For modulation of endogenous DCs, C57BL/6 mice were injected i.v. with 50 µg neo- glycoconjugates and primed one week later by s.c. injection of 200 µg OVA/25 µg poly:IC/25 µg anti-CD40. Seven days after priming, the mice were sacrificed and their spleens were analyzed for the frequency of Tregs and effector T cells. Spleen cells were also re-stimulated overnight with either 2 µg/ml SIINFEKL or 200 µg/ml EKLTEWTSSNMEER OVA peptides in the presence of 5 µg/ml Brefeldin A, then IFN-γ and FoxP3 expression were assessed by intracellular staining using specific antibodies. Alternatively, the cells were re-stimulated in the absence of Brefeldin A to analyze the expression of cytokines in the cell culture supernatants using an ELISA.

Statistical analysis

Prism 5.0 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. The Student's t-test and one-way ANOVA with Bonferroni correction were used to determine statistical significance. Statistical significance was defined

as $P < 0.05$.

Aknowledgements

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Results

Sia-OVA-pulsed DCs promote *de novo* induction of $CD4^+$ $FoxP3^+$ regulatory T cells

As hyper-sialylated pathogens have been shown to negatively modulate the pro-inflammatory functions of APCs, we hypothesized that Sias present on antigens may serve as an inhibitory signal and down-modulate inflammatory T cell responses. Therefore, we examined whether $CD4^+$ T cell polarization is influenced by DCs which have taken up sialylated antigens using well defined synthetic antigen conjugates composed of α2-3- or α2-6-linked Sias chemically linked to the model antigen ovalbumin. The functional presence of α2-3- or α2-6-Sias on Sia- OVA was confirmed by an ELISA using lectins from the plants *Maackia Amurensis* (MAA) and *Sambucus Nigra* (SNA), which are known to have specificity for α2-3- and α2-6-linked Sias, respectively (Figure S1). Subsequently, ex vivo isolated splenic DCs were pulsed with α2-3- or α2-6-Sia-OVA and co-cultured with naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ OT-II T cells. One week later, the T cells were analyzed for the expression of IFN-γ and FoxP3, markers ofeffector and Treg cells, respectively (Zhu et al., 2010). In cultures containing Sia-OVA-pulsed DC, a two- to five-fold increase in $FoxP3^+$ $CD4^+$ T cell number was observed (Figure 1A), while only a small fraction of T cells were polarized into IFN-γ-producing effector T cells. In contrast, T cells primed with native OVA-loaded DCs did not differentiate into $FoxP3^+$ T cells, but differentiated into IFN-γ-producing effector T cells instead (Figure 1A). These data were corroborated by the detection of TNF-α and IFN-γ in the cell supernatants: cultures containing native OVA-loaded DCs contained high levels of both effector T cell cytokines, whereas TNF-α and IFN-γwere virtually absent in cultures containing α2-3- or α2-6-Sia-OVA-DCs (Figure 1B). No significant levels of IL-10 were detected in the Sia-OVA-DC-T-cell co-cultures (data not shown). Comparable results were obtained using bone marrow- derived DCs (BMDCs; Figure S2).

Functional analysis of the induced $FoxP3^+$ $CD4^+$ T cells revealed the presence of suppressive properties (Figure 1C). The presence of suppressive properties was confirmed by flow cytometric analysis of OVA-DC-activated CFSE-labelled OT-II responder T cells (Tresp) co- cultured with $CD4^+$ T cells primed with α2-3- or

α2-6-Sia-OVA-loaded DCs (Tα2-3- or α2-6- Sia-OVA). Addition of T cells primed with either α2-3- or α2-6-Sia-OVA-loaded DCs markedly inhibited the proliferation of Tresp: only a few cells underwent one to two divisions and the majority did not divide (Figure 1C). In contrast, addition of $CD4^+$ T cells primed with native OVA-pulsed DCs (TOVA) did not inhibit the proliferation of Tresp. Under these conditions, and similarly to control cultures to which no T cells were added, T_{resp} were able to undergo up to five cell divisions. As both α2-3- and α2-6-linked Sia-OVA evoked equivalent tolerogenic responses, only the data for α2-6-linked Sia-OVA, designated Sia-OVA, are shown for the remainder of the studies.

Sia-OVA had similar effects on naive T cell polarization in BMDCs isolated from BALB/c mice as C57BL/6-derived DCs: 16% of DO11.10 T cells co-cultured with BMDCs which had taken up Sia-OVA were committed to the $FoxP3^+$ lineage, while almost five-fold fewer T cells became $FoxP3^+$ when co-cultured with OVA-loaded BMDCs (Figure 1D and S3).

DO11.10 T cells primed with OVA-loaded BMDCs became committed to the IFN-γ- producing Th1 lineage. These data indicate that Sia-coupled antigens can induce the maturation of $FoxP3^+$ T cells, irrespective of a Th1- or Th2-stimulatory environment. The induction of $CD4^+$ $FoxP3^+$ T cells using $CD4^+$ T cells from DO11.10 $Rag^{-/-}$ mice, which lack naturally occurring Tregs (Lafaille et al., 1994) demonstrated that the Sia-OVA-DC induced $CD4^+$ $FoxP3^+$ T cells were de novo generated (Figure 1E and S3). Together, these data demonstrate that uptake of Sia-modified antigens by DCs results in the generation of tolerogenic DCs that promote de novo Treg induction and prevent effector T cell generation.

SiglecE mediated internalisation and signalling activation by Sia-OVA result in induction of Tregs by DCs

Induction of Tregs is known to predominantly occur after exposure of $CD4^+$ T cells to low concentrations of antigens, while high concentrations of antigens negatively influence Treg differentiation (Gottschalk et al., 2010). *In vitro* antigen presentation assays suggested that sialylated OVA and native OVA are presented equally in MHC-II molecules, as they induced equivalent levels of proliferation in OVA-specific $CD4^+$ T cells (Figure 2A), leaving us to conclude that Treg

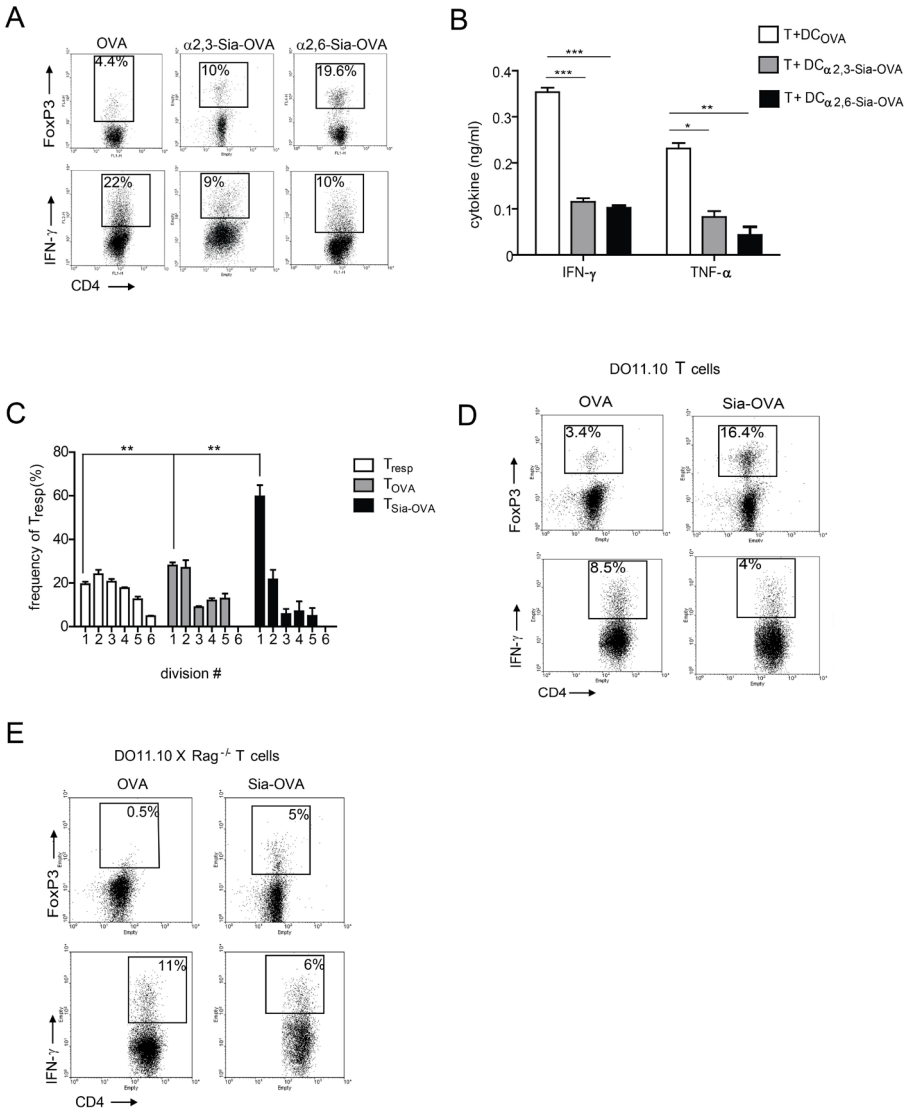

Figure 1. Sia-OVA promotes DC-mediated Treg differentiation and prevents effector T cell generation. (A) Flow cytometric analysis of OT-II T cells differentiated by OVA-, α2,3- Sia-OVA or α2,6-Sia-OVA-loaded DCs. Cells were gated on CD4$^+$ T cells. Numbers in dot plots indicate the percentage of FoxP3$^+$ or IFN-γ$^+$ CD4$^+$ T cells. Dot plots are representative of five independent experiments. (B) Cytometric bead array analysis of cytokines expressed in the supernatants of DC-differentiated OT-II T cells. Data is representative of five independent experiments (mean ± SEM); ***, $P < 0.001$, **, $P < 0.01$; *, $P < 0.05$ (one-way ANOVA with Bonferroni correction). (C) Suppressive activity of T cells primed with OVA-, α2,3-Sia- OVA, or α2,6-Sia-OVA-loaded DCs was examined by addition to CFSE-labeled CD4$^+$ T cells primed with OVA-loaded DCs. After 4 days of culture, the proliferation of the responder cells was determined using flow cytometry. Graph shows the percentage of CFSE-labelled Tresp cells per division. Data are representative of two independent experiments (mean ± SEM); **, $P < 0.01$ (Student's t-test). (D+E) FoxP3 and IFN-g expression by DO11.10 and DO11.10xRag$^{-/-}$ CD4$^+$ T cells after 6 days co-culture with Sia-OVA- or OVA-loaded DCs. Cells were gated on CD4$^+$ T cells. Numbers in dot plots indicate the percentage of FoxP3$^+$ or IFN-γ$^+$ CD4$^+$ T cells. Data are representative of two independent experiments (mean ± SEM).

induction by Sia-OVA-loaded DCs was not the result of exposure to low levels of antigen on the DC surface. This suggestion was further substantiated by the observation that modification of OVA with Sias did not affect OVA uptake by DCs (Figure 2B). In fact, higher amounts of Sia-OVA than native OVA were taken up by DCs.

In addition,titration experiments revealed that DCs loaded with very low amounts of Sia-OVA (i.e. $\leq$ 5 µg/ml) did not promote FoxP3$^+$ CD4$^+$ T cell induction to a greater extent than DCs loaded with equal amounts of native OVA (Figure 2C). Notably, at this low antigen concentration, a clear inhibition of effector T cell generation was observed.

The effects observed on T cell responses were highly specific for Sias; for example, significant FoxP3$^+$ Treg induction was not observed when DCs were loaded with OVA coupled to glycans that target C-type lectin receptors such as the mannose receptor (i.e. OVA- GlcNAc)(Singh et al., 2011). Similarly to Sia-OVA, modification of OVA with GlcNAc resulted in improved internalization of OVA (Figure S4A). However, DCs loaded with OVA- GlcNAc promoted the differentiation of naive CD4$^+$ T cells into Th1 cells and not FoxP3$^+$ Treg (Figure S4B).

Thus, while our data demonstrate that antigens modified with Sias are efficiently taken up by DCs, it remained unclear how these Sia-antigens relay tolerogenic signals within DCs. We hypothesized that siglecs may play a role in the binding and internalisation of Sia-OVA, as well as potentially triggering a signaling cascade that induces modulation of the DC phenotype (Crocker et al., 2007). As the CD33-related Siglec-E is expressed on immature murine DCs and is known to preferentially bind α2-3- and α2-6-linked Sias (Crocker et al., 2007), we investigated whether Siglec-E could be the receptor on immature murine DCs that binds Sia-OVA. Comparison of the binding and uptake of Sia-OVA by Siglec-E$^{-/-}$ DCs and wild-type DCs revealed that both processes were substantially reduced in Siglec-E$^{-/-}$ DCs (Figure 3A). Furthermore, we found that the absence of Siglec-E on DCs profoundly arrested CD4$^+$ FoxP3$^+$ T cell induction in response to Sia-OVA (Figure 3B): 15% of CD4$^+$ T cells differentiated into FoxP3$^+$ T cells when co-cultured with Sia-OVA-loaded wild-type DCs compared to only 0.5% in cultures containing Sia-OVA-loaded Siglec-E$^{-/-}$ DCs. Moreover, Siglec-E$^{-/-}$

DCs did not prevent the generation of IFN-γ-producing effector $CD4^+$ T cells. In fact, Siglec-$E^{-/-}$ DCs loaded with either native OVA or Sia-OVA equally skewed naive $CD4^+$ T cells towards IFN-γ-producing effector cells (i.e. 13% vs. 11%, respectively, Figure 3B).

Together, these data suggest that Sia-OVA predominantly imposes tolerogenic function on DCs via its interaction with Siglec-E on the DC surface. Having established that Siglec-E- mediated uptake of Sia-modified antigens endows DCs with tolerogenic capacities, we next assessed whether the binding of Sias alone is sufficient to provide DCs with tolerogenic capacity. DCs were incubated with

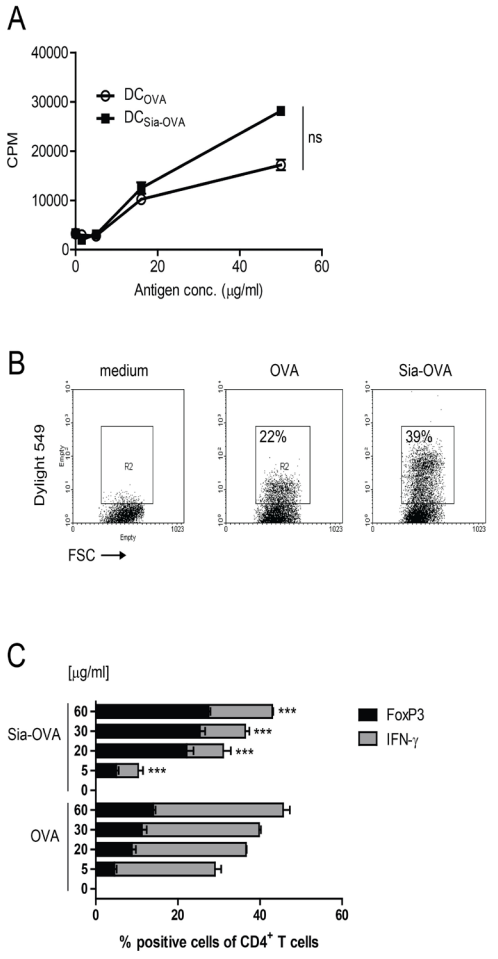

Figure 2. Sia-OVA-DC-mediated Treg generation is antigen dose-dependent. (A) Antigen presentation in MHC-II was analysed by assessing the proliferation of OT-II T cells as determined by [^{3}H]thymidine incorporation. Data are representative of three independent experiments (mean ± SEM); ns, not significant (Student's t-test). (B) DCs were incubated with Dylight549-labeled-OVA or Dylight549-labeled-Sia-OVA for 2 h then antigen uptake was analysed using flow cytometry. Data are representative of three independent experiments. (C) Naive OT-II T cells were differentiated by DCs which had been pulsed with the indicated concentrations of Sia-OVA or unmodified OVA. Graphs indicate the percentage of $FoxP3^+CD4^+$ and IFN-γ^+CD4^+ T cells for each antigen concentration tested. Data are representative of three independent experiments (mean ± SEM). Statistical significance for differentiation with 20, 30 and 60 mg/ml antigen-loaded DCs is indicated; ***, $P < 0.001$ (Student's t-test).

a mix of Sias and OVA antigen and then subsequently co- cultured with OVA-responsive T cells. DCs only gained tolerogenic properties when the Sias were covalently coupled to OVA, and not when the Sias and OVA were provided separately, as indicated by high levels of effector T cell expansion in the cultures containing DCs loaded with a mixture of OVA and Sias (Figure 3C).

Surface marker analysis of the Sia-OVA-pulsed DCs did not unveil a "classic" tolerogenic phenotype, as the expression of co-stimulatory and MHC-II molecules on DCs loaded with Sia-OVA was similar to native OVA-pulsed DCs. Furthermore, no distinct or enhanced expression of markers associated with Treg induction, such as PD-L1, PD-L2, B7-H4 (Manicassamy and Pulendran, 2011), were detected on Sia-OVA-loaded DCs (data not shown). However, analysis of LPS-induced cytokine production by antigen-pulsed DCs revealed the presence of lower amounts of pro-inflammatory cytokines in the supernatant of Sia-OVA-loaded DCs than native OVA-loaded DCs (Figure 3D), and no significant differences in IL-10 secretion were observed. To better assess whether the tolerogenic effects of Sia-OVA-loaded DCs occur in a soluble factor-dependent manner, we incubated OVA- loaded DCs and naive OT-II T cells with the supernatants from Sia-OVA-DC cultures. In this case, OVA-loaded DCs did not acquire tolerogenic and suppressive effects during T cell differentiation. Similar results were observed when the OVA-loaded DC-T cell co-culture was separated from Sia-OVA-loaded DCs using Transwell chambers (Figure 3E). Overall, our data strongly suggests that the induction of Tregs and prevention of effector T cell generation by Sia-OVA-targeted DCs could involve a membrane-bound receptor-ligand interaction. Together, these data indicate a relevant role for Siglec-E in the tolerogenic effect of Sia- antigens on DCs. Moreover, Sia-antigen-loaded DCs mediate tolerance in a cell-cell contact- dependent fashion. However, no "classic" tolerogenic phenotype was detected in DCs upon Siglec-E-mediated Sia-OVA uptake.

Treatment of mice with Sia-antigen dampens effector T cell responses

We next evaluated the tolerogenic capacity of Sia-OVA-loaded DCs *in vivo*. Accordingly, Sia-OVA-pulsed DCs were adoptively transferred into C57BL/6

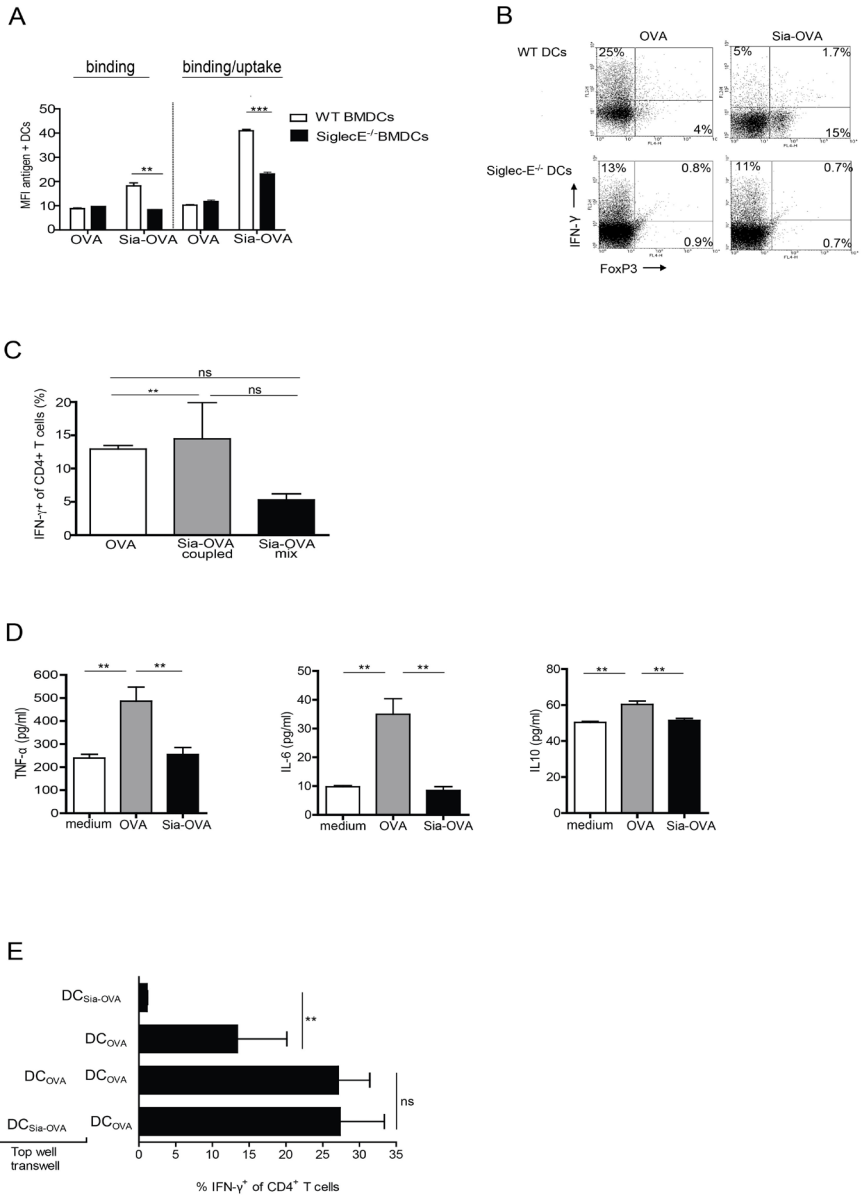

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Figure 3. Siglec-E on DCs is involved in Sia-OVA binding and uptake, as well as Treg generation. (A) Binding and internalization of Dylight549-labeled Sia-OVA or OVA by wild-type (WT) and SiglecE$^{-/-}$ DCs, as determined by flow cytometry. Graph shows the mean fluorescence intensity (MFI) of bound or internalized antigen. Means ± SEM of four independent experiments are indicated; **, $P < 0.01$; ***, $P < 0.001$ (Student's t-test). (B). Flow cytometric analysis of FoxP3 and IFN-γ expression in OT-II T cells differentiated by antigen-loaded WT and SiglecE$^{-/-}$ BMDCs. Cells were gated on CD4^{+} T cells. Numbers in dot plots indicate the percentage of gated cells in the respective quadrants. Dot plots are representative of two independent experiments. (C) BMDCs were loaded with either unmodified OVA, Sia-OVA, or a mix of OVA and Sias, and then subsequently co-cultured with naive OT-II T cells. Frequencies of IFN-γ-producing effector OT-II T cells were determined by IC-cytokine staining. Data

show the means ± SEM of three independent experiments; **, P < 0.01; ns, not significant (Student's t-test). (D) Secretion of TNF, IL-6, and IL-10 by DCs incubated with OVA or Sia-OVA in the presence of LPS were analysed using ELISAs. Cytokines were measured in pg/ml. Means ± SEM of five independent experiments are shown; **, P < 0.01 (Student's t-test). (E) The potential involvement of a soluble factor in tolerance induction by Sia-OVA-loaded DCs ($DC_{Sia\text{-}OVA}$) was tested by separating $DC_{Sia\text{-}OVA}$ (top well) from co-cultures of OVA-pulsed DCs (DC_{OVA}) and naive OT-I T cells (lower cells) using transwell inserts. On day 7, OT-II T cells were analyzed for IFN-γ^+ cells using flow cytometry. The mean ± SEM percentages of IFN-γ^+ $CD4^+$ T cells of triplicate wells are presented. This is a representative of four independent experiments; **, P<0.01; ns, not significant (Student's t-test).

mice to examine inhibition of subsequent effector T cell generation. Compared to control mice that received OVA-loaded or non-antigen-loaded DCs, reduced frequencies of effector T cells were observed in mice that received Sia-OVA-loaded DCs, as revealed by significantly reduced numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells (Figure 4A). This was mirrored by lower amounts of TNF-α and IFN-γ in the culture supernatants of in vitro re-stimulated splenocytes (Figure 4B). Importantly, the reduction in effector T cell numbers observed in mice pre-treated with Sia- OVA-DCs was accompanied by significantly higher frequencies of $CD4^+$ $FoxP3^+$ T cells (Figure 4C). As these data confirmed that in vitro-generated Sia-OVA-loaded DCs maintain the capacity to induce suppressive Tregs in vivo, we continued to investigate whether administration of Sia-OVA could modulate endogenous DCs. For this purpose, C57BL/6 mice were injected with Sia-OVA one week prior to immunization with OVA, anti-CD40 and poly(I:C). We observed that pretreatment of the mice with Sia-OVA dampened subsequent induction of effector T cell formation, as indicated by the lower proportions of IFN-γ producing $CD8^+$ T cells detected ex vivo (Figure 5A). This inhibitory effect was not observed in control mice which received native OVA or PBS. Notably, compared to control mice, inhibition of effector T cell expansion in mice injected with Sia-OVA was accompanied by a higher frequency of $CD4^+$ $FoxP3^+$ T cells (Figure 5B). Thus, DCs also become tolerogenic in vivo upon uptake of Sia-OVA, resulting in Treg expansion and effector T cell dampening.

Sia-antigen also relays tolerogenic properties to DCs under inflammatory conditions

In the experiments described above, DCs were modulated by Sia-OVA under homeostatic conditions. To determine whether Sia-OVA also relays tolerogenic function to DCs under inflammatory conditions, we assessed naive $CD4^+$ T cell

differentiation by DCs pulsed with Sia-OVA or native OVA in the presence of pro-inflammatory signals such as LPS or CpG. While naive $CD4^+$ T cells markedly differentiated into effector T cells when primed with OVA/LPS-pulsed

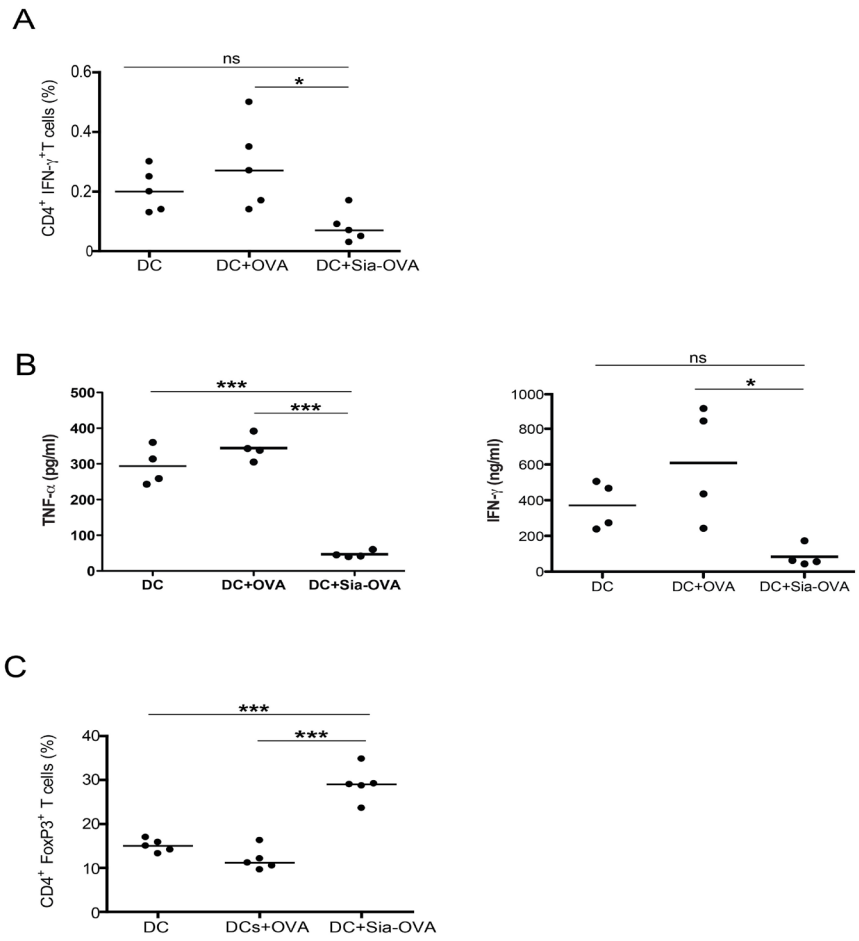

Figure 4. Adoptive transfer of Sia-OVA-loaded DCs into mice promotes Tregs and prevents affector immunity. Antigen-loaded or unloaded control DCs were adoptively transferred into C57BL/6 mice. One week later, the mice were boosted using OVA mixed with CpG. Seven days later, the spleens were collected and analysed for $FoxP3^+$ and IFN-γ-expressing $CD4^+$ T cells. (A) Percentage of IFN-γ^+ $CD4^+$ T cells among total splenocytes as determined by flow cytometry. Dots represent individual mice (n=5 per group) and the bars indicate the median; *, P< 0.05; ns, not significant (one-way ANOVA with bonferroni correction). (B) Cytometric bead array analysis of cytokines secreted by the splenocytes of treated micewhich were re-stimulated in vitro with PMA/ionomycin (n=5 per group). Data are mean ± SEM; , P<0.05;*, P< 0.001; ns, not significant (one-way ANOVA with Bonferroni correction). (C) Flow cytometric analysis of $CD4^+$ $FoxP3^+$ T cells among $CD4^+$ splenocytes. Dots represent individual mice (n=5 per group) and the bars indicate the edian; ***, P<0.001 (one-way ANOVA with Bonferroni correction).

DCs, substantial Treg induction and controlled effector T cell generation also occurred when DCs were targeted with Sia-OVA under inflammatory conditions, as indicated by intracellular staining for FoxP3 and IFN-γ, and TNF-α and IFN-γ ELISAs of the re-stimulated T cell supernatants (Figure 6A and B). Similar results were obtained when LPS-treated mature DCs were targeted with Sia-OVA (data not shown). Sia-OVA-loaded DCs also dampened effector T cell formation when co-cultured with OVA-loaded DCs and OT-II T cells (Figure 6C).

These data suggest that targeting DCs with Sia-modified antigens might be powerful enough to dampen ongoing inflammatory immune reactions. To test this hypothesis, we modified a peptide derived from myelin oligodendrocyte glycoprotein (MOG) which is a known target of autoreactive T cells in experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis (Nylander and Hafler, 2012). Subsequently, DCs loaded with α2-6-linked Sia-

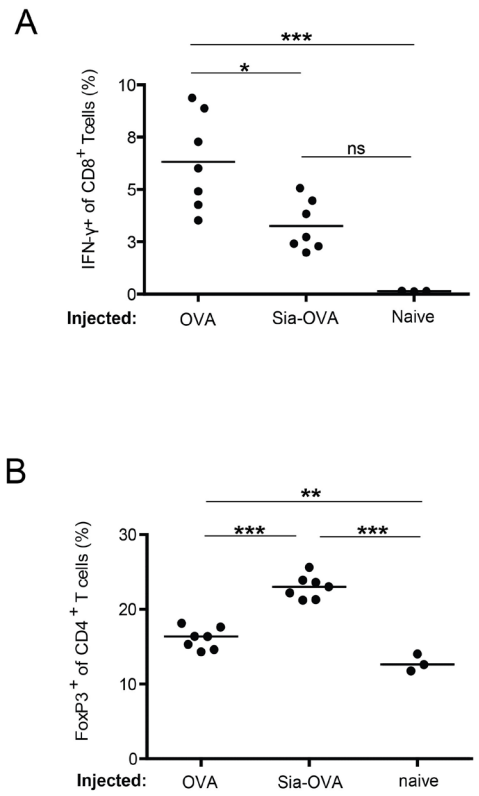

Figure 5. Sia-OVA induces tolerance and prevents the effector immune response *in vivo*. C57BL/6 mice received OVA or Sia-OVA one week prior to sensitization with OVA mixed with Poly I:C and agonistic anti-CD40 antibody. Naïve mice were assessed as controls. One week later, splenocytes were analyzed. (A) Frequencies of IFN-γ+ CD8+ T cells among total splenocytes. (B) Percentage of CD4+ FoxP3+ T cells among total splenocytes as determined by flow cytometry. Dots represent individual mice (n=7 per group) and the bars indicate the median; *, P<0.05; ***, P<0.001; ns, not significant (one-way ANOVA with Bonferroni correction).

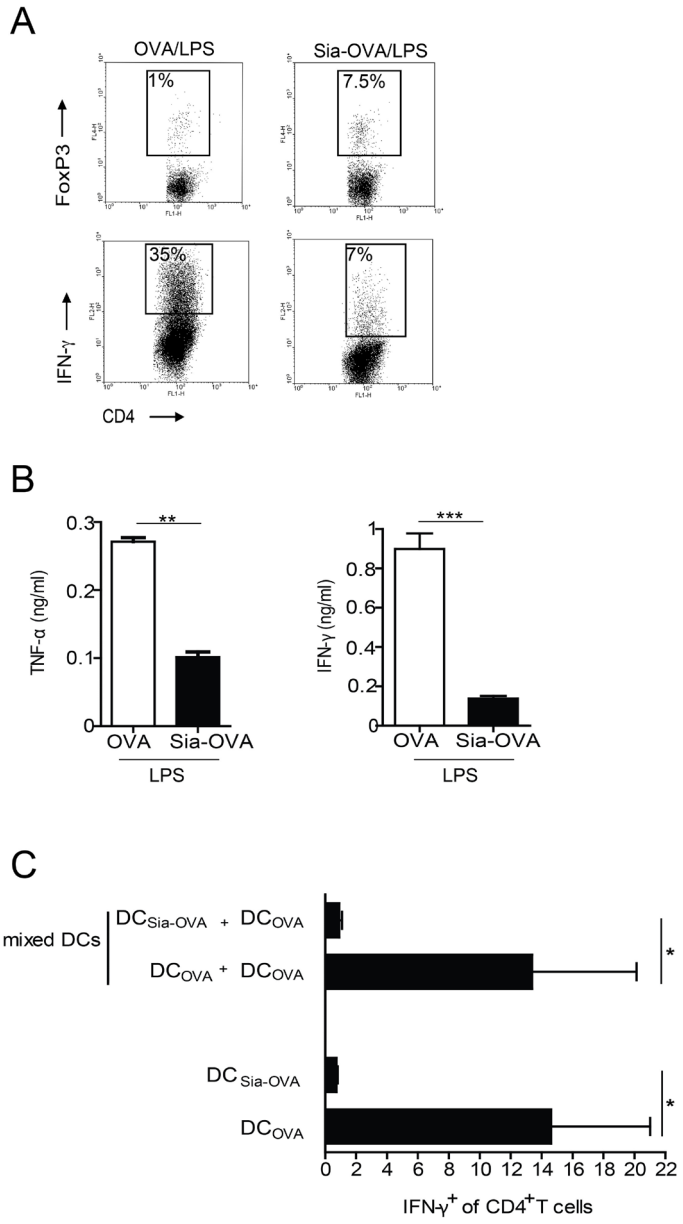

Figure 6. Sia-OVA also modulates DCs in an inflammatory environment. (A) Flow cytometry analysis of OT-II T cells co-cultured with DCs which had been loaded with antigens in the presence of LPS. Dot plots are gated on $CD4^+$ T cells; the numbers in the dot plots indicate the percentage of $CD4^+$ $FoxP3^+$ or $CD4^+$ IFN-γ^+ cells. (B) The levels of TNF and IFN-γ in the supernatants of the cultures described in (A) were determined by ELISAs. Data indicate the means ± SEM of five independent replicates; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (Student's t-test). (C) DCs were loaded with Sia-OVA ($DC_{Sia\text{-}OVA}$) or OVA (DC_{OVA}) for 4 h and mixed 1:1 with DC_{OVA} before co-culture with naïve OT-II T cells. Cultures containing only $DC_{Sia\text{-}OVA}$ or DC_{OVA} were used as controls. On day 6, OT-II T cells were analyzed for the proportion of IFN-γ^+ $CD4^+$ T cells by flow cytometry. Data shown are the mean ± SEM of one out of three independent experiments;*, $P>0.05$ (Student's t test).

MOG_{35-55} (Sia-MOG) or native MOG_{35-55} were used to re-stimulate splenocytes from mice with different EAE clinical scores, which represent different stages of effector T cell number and activity. We observed that MOG_{35-55}-loaded DCs induced proliferation and IFN-γ production by splenocytes collected from mice which were at the onset of disease (score 1) as well as mice at a more severe phase of the disease (scores 2 or 3). Notably, proliferation and IFN-γ production by splenocytes were significantly suppressed when using Sia-MOG-loaded DCs (Figure 7A and B). As total splenocytes were incubated with the Sia-MOG-loaded DCs for four days in this experiment, it is likely that the observed suppression was not mediated via de novo induced $CD4^+$ Tregs. To assess whether Sia-modified MOG endows DCs with Treg-inducing properties, we monitored the differentiation of naive MOG_{35-55}–responsive $CD4^+$ T cells derived from 2D2 TCR transgenic mice (Bettelli et al., 2003). DCs loaded with native MOG_{35-55} peptide promoted the differentiation of naive 2D2 T cells into Th1 effector cells, as indicated by the production of IFN-γ and expression of T-bet mRNA (Figure 7C and D). In contrast, reduced frequencies of Th1 cells were observed in cultures with DCs that had taken up Sia-MOG. Importantly, these Sia-MOG-loaded DCs promoted the induction of FoxP3 mRNA and protein expression in the 2D2 $CD4^+$ T cells (Figure 7C and E). These data indicate that Sia-modification of antigens alters their immunogenicity and could potentially be used to dampen detrimental immune reactions.

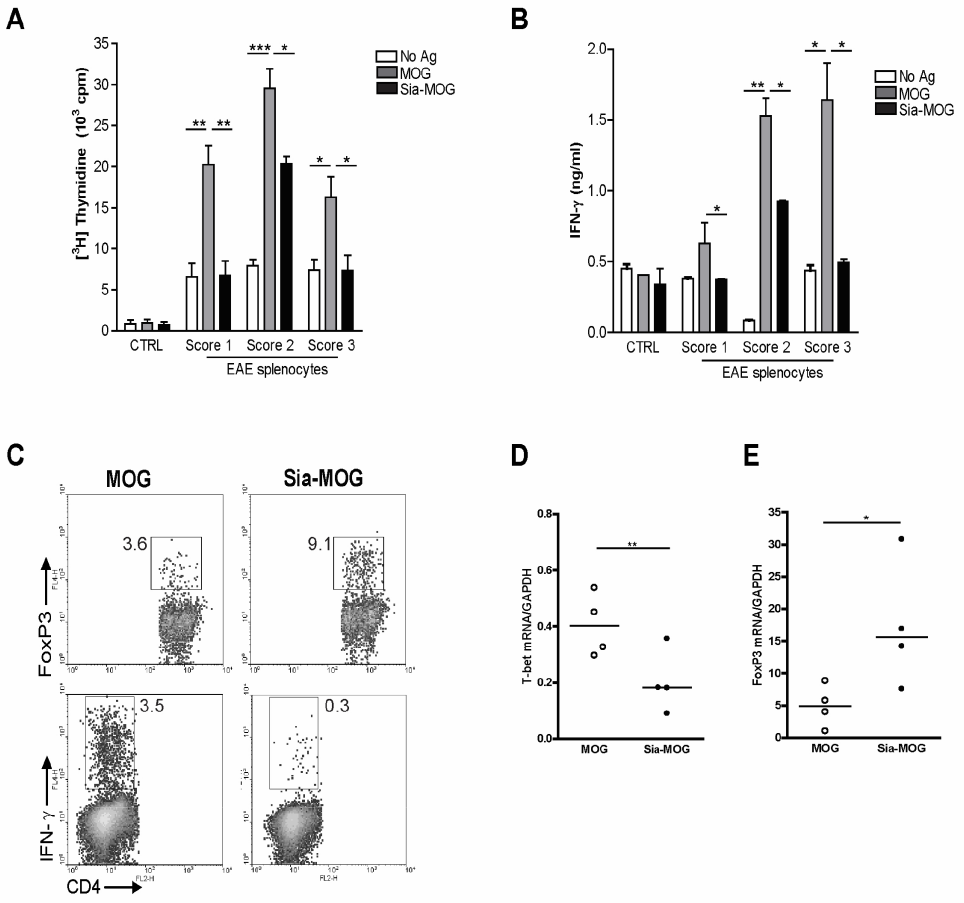

Figure 7. Treg induction and dampening of autoreactive T cell activity by DCs targeted with Sia-MOG. (A, B) The spleens of mice suffering from experimental autoimmune encephalomyelitis (EAE) were re-stimulated *in vitro* with DCs loaded either with Sia-MOG (black bars) or native MOG$_{35-55}$ (grey bars). As a control, PBS-treated DCs (white bars) and the spleens of naive mice were assessed. On day 4 of culture, [3H] thymidine was added, incubated for 18 h, and then (A) MOG-specific T cell proliferation was determined and (B) IFN-γ was detected in the culture supernatants using an ELISA. Data shown are the means of triplicate cultures from three animals per condition. (C) Naive 2D2 T cells were cocultured with DCs loaded with Sia-MOG or unmodified MOG. To some cultures 2 ng/ml TGF-β was added. After 6 days, the DC-T cell co-culture was analysed for the proportion of FoxP3$^+$ and IFN-γ$^+$ cells using flow cytometry. Additionally, the expression of (D) T-bet and (E) FoxP3 mRNA were determined using qRT-PCR. Gene expression was normalized and presented as relative expression to GAPDH. Values shown are the means ± SEM of triplicate culture.

Discussion

Here we show, for the first time, that DCs become tolerogenic upon uptake of soluble sialylated antigens. DCs which had taken up Sia-modified antigens primed antigen-specific T cells to become suppressive FoxP3$^+$ Tregs while simultaneously limiting the expansion and function of effector T cells. Additionally, we demonstrated a critical role for Siglec-E in mediating the tolerogenic effects of Sia-OVA. Importantly, the tolerogenic effect of Sia-OVA was maintained under inflammatory conditions. Titration of Sia-antigen revealed that while low concentrations of Sia-antigen were sufficient to inhibit effector T cell expansion, they did not promote the induction of Tregs, suggesting that different mechanisms underlie the two tolerogenic effects of Sia-OVA.

Moreover, we provide evidence for induction of antigen specific immune tolerance under inflammatory conditions using sialylated antigens. We observed that under inflammatory conditions effector T cell functions were also dampened by Sia-OVA-loaded DCs. Importantly, under these circumstances Sia-OVA-loaded DCs retained the capacity to polarize naive CD4$^+$ T cells towards Tregs. To the best of our knowledge, this sustained tolerizing effect has not been demonstrated for other tolerizing compounds. The possibility of tolerizing DCs in vivo in an antigen specific manner via this new strategy of sialylation of antigens may open up new avenues for treating patients who suffer from unwanted immune reactions such as autoimmunity and allergies.

Our findings indicated that the differentiation of naive CD4$^+$CD25$^-$ T cells into Tregs and reduced generation of effector IFN-γ^+ T cells by Sia-OVA-pulsed DCs were not dependent on the mouse strain, as DCs and T cells from both C57BL/6 and BALB/c mice underwent equivalent responses. Additionally, we even observed Treg differentiation using naive CD4$^+$ T cells from DO11.10 Rag$^{-/-}$ mice, which lack naturally occurring Tregs (nTregs), suggesting that Sia-OVA-pulsed DCs induce de novo generation of Tregs. However, we anticipate that DCs tolerized by the uptake of Sia-OVA *in vivo* may also propagate nTregs, as the detection of FoxP3$^+$ CD4$^+$ T cells does not discriminate between iTregs and nTregs. Nevertheless, the net result of Sia-antigen treatment is the formation of increased numbers of T cells with suppressive capacity which can inhibit

detrimental effector T cell responses.

Our studies further showed that Sia-OVA-DC-induced Tregs clearly suppressed the proliferation of responder T cells. However, how these Tregs arrest T responder proliferation is unknown and was beyond the scope of this study. Tregs have been reported to suppress immune responses and/or maintain immune homeostasis by different mechanisms. These include the production of inhibitory cytokines such as IL-10 and TGF-β, cell-contact-dependent suppression, and metabolic disturbance (Sakaguchi et al., 2009). In our studies, we did not detect a significant increase in either IL-10 or TGF-β in the supernatants of Sia-OVA-DC-induced $FoxP3^+$ T cells. Hence, it is possible that Sia-OVA-DC-induced Tregs suppress rthe effector immune response in a cell-contact-dependent manner. Analysis of the Sia-OVA- pulsed DC phenotype did not reveal significant differences in the expression of co-stimulatory molecules such as CD80, CD86, PD-L1 and PD-L2. In addition, the expression of MHC-II on Sia-OVA-DCs was similar to OVA-loaded-DCs, excluding the possibility that Tregs are induced by reduced antigen presentation following uptake of Sia-OVA (Kretschmer et al., 2005). We previously observed a similar absence of clear effects on the phenotype of human monocyte-derived DCs after incubation with the highly sialylated pathogen N. gonorrhoea, although T cell polarizing capacity was affected (Bax et al., 2011; van Vliet et al., 2009). Despite producing lower amounts of the pro-inflammatory cytokines IL-6 and TNF, we did not observe significant differences in the quantity of IL-10 or TGF-β produced by Sia-OVA- loaded DCs compared to native OVA-loaded DCs. Therefore, it is possible that Sia-OVA- pulsed DCs induce Treg induction and prevent effector T cell generation via cell-surface receptor-ligand interactions, rather than secretion of anti-inflammatory cytokines. This suggestion was further substantiated by our findings that Sia-OVA-loaded DCs did not confer tolerogenic capacity onto $CD4^+$ T cells when physically separated or when the supernatant of Sia-OVA-loaded DCs was used to treat native OVA-loaded DCs.

It has been conclusively demonstrated that Sias present on pathogens inhibit the innate immune response by binding to siglecs expressed on innate immune cells (Crocker et al., 2007). Many siglecs act as negative regulators of immune responses via the expression of ITIM motifs in their cytoplasmic tails. Therefore,

it is likely that the observed tolerogenic effect of Sia-antigen is the result of an interaction of Sia-antigen with a siglec receptor present on DCs. Siglec-E is an ITIM-containing receptor which has been described to negatively modulate the cytokine response of DCs (Boyd et al., 2009). Our studies showed a significant reduction in the binding and uptake of Sia-OVA by Siglec-$E^{-/-}$ DCs, which suggests a relevant role for this receptor in the interaction of Sia-OVA with DCs. Concomitant with reduced binding and uptake of Sia-OVA, we found that the incubation of Siglec-$E^{-/-}$ DCs with Sia-OVA drastically inhibited Treg generation, but did not affect $CD4^+$ T cell differentiation into IFN-γ-producing effector T cells. In fact, Sia-OVA-Siglec$E^{-/-}$ DC-primed T cells were equally skewed towards IFN-γ-producing effector cells as T cells stimulated with native OVA-loaded Siglec$E^{-/-}$ DCs. In view of these findings, Sia-OVA-mediated Treg induction and T effector inhibition may occur via two different modes, with Siglec-E mainly implicated in Treg polarization. Additionally, pulsing wild-type DCs with low concentrations of Sia-OVA antigen did not lead to enhanced generation of Tregs, but still dampened effector T cell generation. It can be speculated that low antigen concentrations do not evoke clustering of Siglec-E, which is necessary in order to relay intracellular ITIM signalling[1].However, the molecular mechanisms, as well the potential contribution of siglecs, to Sia- OVA-mediated tolerogenic effects need to be investigated in more detail.

In healthy individuals, the most important role of Tregs is to maintain immune tolerance to self and innocuous exogenous antigens as well as the intestinal microflora, to prevent the development of auto-immune and allergic diseases (Sakaguchi et al., 2010). Defects in Treg cell number and/or function have been shown to contribute to auto-immune diseases (Buckner, 2010). Thus, therapies directed at resolving Treg defects have the potential to prevent and also cure such diseases. Modification of specific antigens using Sias has a number of advantages over current therapeutic strategies aimed at establishing large cohorts of Tregs: it provides DCs with dual tolerogenic function, as in addition to induction of Tregs, Sias simultaneously inhibit the generation of IFN-γ-producing T cells. Thus, such a novel therapy may directly dampen excessive inflammation, a process which occurs during autoimmunity. Furthermore, in contrast to current adjuvant approaches such as administration of retinoic acid or TGF-β, non-specific

suppression is minimized as the specific antigen is directly modified with Sias. In conclusion, our data demonstrate that sialylation alters the immunogenicity of an antigen and provides a novel way to induce tolerogenic DCs for the treatment of autoimmune diseases and allergies.

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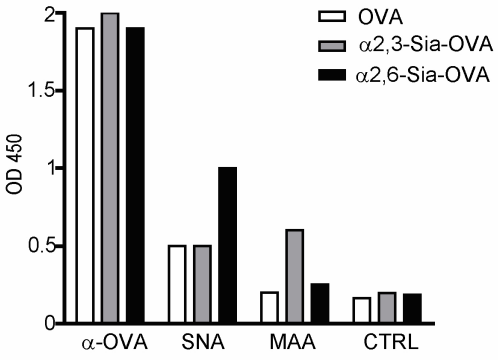

Figure S1. Detection of α2,3- or α2,6-Sia-modified antigens. Antigens were coated and the presence of α2-3- or α2-6-linked Sias was detected by ELISA using the *Maackia Amurensis* (MAA) and *Sambucus Nigra* (SNA) derived lectins, respectively. Additionally, OVA was detected using an anti-OVA antibody. Data are mean ± SEM of duplicate measurements and representative of five independent experiments.

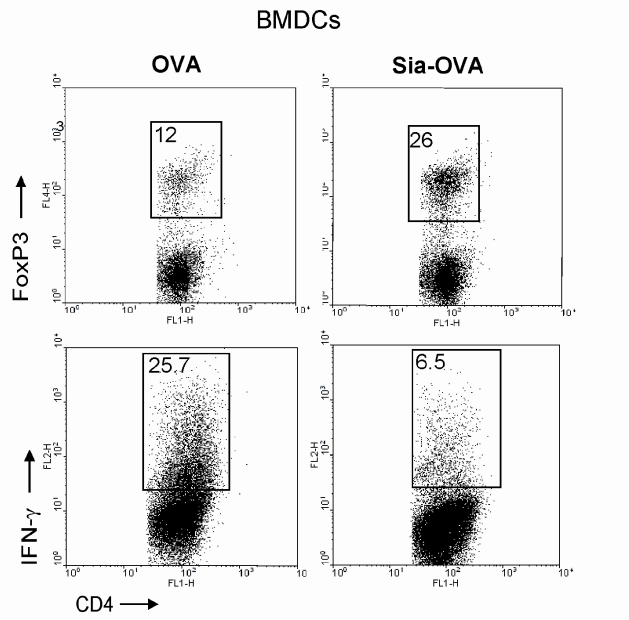

Figure S2. Sia-OVA imposes tolerogenic features on bone-marrow derived DCs. Flow cytometric analysis of OT-II T cells after 6 days co-culture with Sia-OVA- or OVA-loaded BMDCs. Cells were gated on $CD4^+$ T cells. Numbers in dot plots indicate the percentage of $FoxP3^+$ or IFN-γ^+ $CD4^+$ T cells. Values plotted are the means ± SEM and representative of five independent experiments.

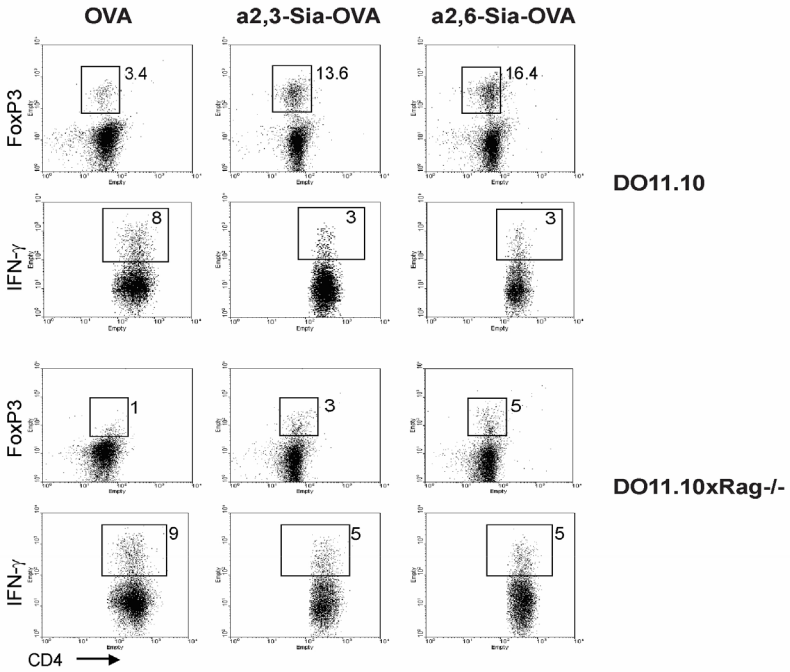

Figure S3. Equal induction of Tregs by OVA modified with α2,3- or α2,6-Sias. (A and B) Flow cytometric analysis of (A) DO11.10 and (B) DO11.10 X $Rag^{-/-}$ $CD4^+$ T cells after co-culture with OVA, α2,3-Sia-OVA, or α2,6-Sia-OVA-loaded DCs. Cells were gated on $CD4^+$ T cells. Numbers in dot plots indicate the percentage of $FoxP3^+$ or IFN-γ^+ $CD4^+$ T cells. Dot plots are representative of two independent experiments.

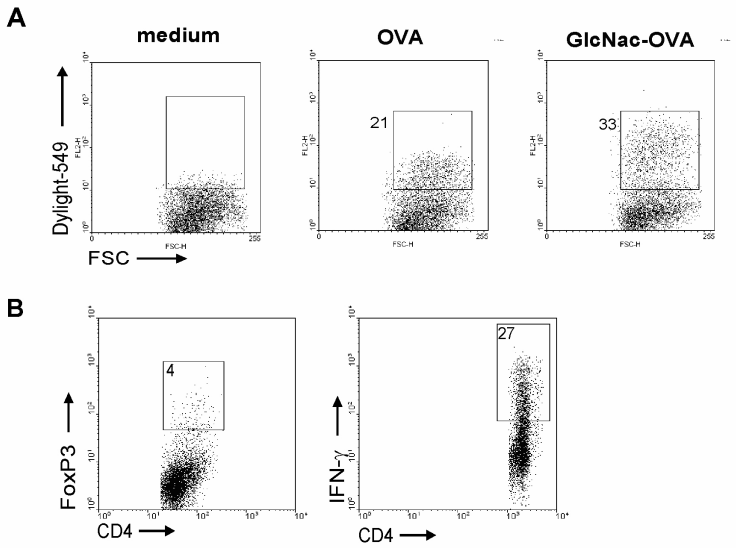

Figure S4. OVA modified with mannose receptor binding glycans promotes Th1 differentiation. (A) DCs were incubated with Dylight549-labeled OVA or GlcNac-OVA, which targets the mannose receptor, for 2 h at 37°C. Antigen binding/uptake was analysed using flow cytometry. Values shown are the means ± SEM of three independent experiments. (B) OVA-specific $CD4^+$ T cells were co-cultured with OVA- or GlcNac-OVA-loaded DCs and one week later the frequencies of $FoxP3^+$ and IFN-γ^+ $CD4^+$ T cells were determined using flow cytometry. Data representative of three independent experiments are shown.

above: Caravaggio; *The Conversion on the Way to Damascus* (1601). Santa Maria del Popolo, Rome.

Chapter 4

Generation of CD8$^+$ regulatory T cells by loading dendritic cells with sialic acid-modified antigens

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Manuscript in preparation

Abstract

Dendritic cells (DCs) provide essential signals that induce that induce the differentiation of naive $CD8^+$ T cells naive T cells into lytic effector cells, needed to clear viral infections or tumor cells. We previously demonstrated that upon uptake of sialic acid (Sia)-modified antigens DCs become tolerogenic and instruct naive $CD4^+$ T cells to differentiate into regulatory $CD4^+$ T cells. However, whether Sias on antigens affect the signals DCs provide to $CD8^+$ T cells is unknown. We here show that DCs that have taken up Sia-modified antigens strongly inhibited the expansion of naive $CD8^+$ T cells and impaired the acquisition of cytotoxic effector properties as indicated by lower frequencies of IFN-γ as well as Granzyme B-producing $CD8^+$ T cells compared to T cells activated by native OVA-loaded DCs. A proportion of the $CD8^+$ T cells activated by Sia-OVA-loaded DCs became $FoxP3^+$. Additionally, these T cells showed distinct and high expression of CD39, but not GITR and CTLA-4. Induction of tolerogenic $CD8^+$ $FoxP3^+$ T cells was abrogated when DCs ingested sialylated antigens in the presence of the TLR4 ligand lipopolysaccharide. By contrast, cytotoxic effector $CD8^+$ T cell formation was still prevented by DCs loaded with Sia-antigen loaded under this condition. Expression of co-stimulatory receptors known to mediate $CD8^+$ T cell activation was not significantly different between DCs pulsed with Sia-OVA or native OVA, leaving a potential mechanism of the tolerogenic DCs unveiled. In conclusion, our studies show that uptake of sialylated antigens tolerizes the $CD8^+$ T cell activating and differentiation capacity of DCs, having thus therapeutic implications in diseases characterized by inappropriate $CD8^+$ T cell responses.

Introduction

Cytolytic $CD8^+$ T lymphocytes (CTLs) provide a strong and efficient response against virus infection and tumor formation. CTLs specifically recognize antigen-loaded MHC I molecules expressed on virally infected or tumor cells. This interaction triggers CTLs to release IFN-γ and TNF-α, as well as Granzyme B (GrB) and perforin to efficiently kill the target cell[1-3]. The generation of a large pool of virus and tumor-specific CTLs depends on the interaction of naive $CD8^+$ T cells with dendritic cells (DCs)[4]. The crucial signals that induce naive $CD8^+$ T cell activation and differentiation provided by DCs include display of high levels of antigen- loaded MHC class I molecules and co-stimulatory proteins as well as the secretion of inflammatory cytokines[5]. Important co-stimulatory molecules shown to be critical for $CD8^+$ T cell expansion and survival include CD80, CD86, CD40, CD70 and 4-1BB[6,7].

DCs display receptors on their surface that recognize carbohydrate structures on membrane- expressed proteins and lipids of viruses and transformed cells. Among these receptors are sialic acid-binding lectins (Siglecs). Siglecs specifically recognize sialic acids (Sias), which are the outermost sugars present on cell surface expressed proteins and lipids. Most Siglecs contain Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in their intra-cellular portion, which mediate an inhibitory negative signaling cascade that modulates cell functions when Siglecs are bound by Sias[8]. Indeed, Siglecs are known as negative regulators of the activation and function of DCs as well as macrophages and Natural Killer (NK) cells[9]. The interaction of sialylated pathogens with Siglecs on DCs and macrophages has been shown to impair the secretion of pro-inflammatory cytokines IL-6 and IL-12[10,11]. In addition, Siglec triggering affects the $CD4^+$ T cell polarization capacity of DCs. We have shown that lipooligosaccharides (LOS) structures of C. Jejuni divert human DC mediated $CD4^+$ T cell differentiation towards either the T helper1 (Th1) or T helper 2 (Th2) lineage, depending of the type of Siglec involved in recognizing these structures[12]. Recently, we showed that the uptake of sialylated antigens converts DCs into tolerogenic DCs, which promote the generation of $CD4^+$ $FoxP3^+$ regulatory T (Tregs) cells and prevent effector $CD4^+$ T cell formation (Perdicchio et al., manuscript submitted).

Tregs play a crucial role in the development and maintenance of immunological tolerance by preventing excessive and unwarranted immune reactions. Different types of Tregs have been identified and characterized. The most well-defined Tregs are the thymus-derived $CD4^+$ Tregs, known as naturally occurring Tregs (nTregs), which highly express the IL-2 receptor a-chain (CD25) and constitutively express the transcription factor Forkhead box P3 (FoxP3). However, Tregs can be also induced in vitro or in vivo (iTregs) under particular conditions of antigenic stimulation. Besides CD25 and FoxP3, also cytotoxic T-lymphocyte antigen-4 (CTLA4), Glucocorticoid-Induced TNFR-Related (GITR) protein, Glycoprotein A repetitions Predominant (GARP) and the ectonucleotidases CD39 and CD73 have been associated with nTregs and iTregs[13-15]. Besides $CD4^+$ Tregs, $CD8^+$ nTregs and $CD8^+$ Tregs have also been identified.

At tumor sites, the frequency of both $CD4^+$ and $CD8^+$ nTregs and iTregs tend to highly increase, interfering with anti-tumor immune response[16]. In the microenvironment of many tumors such as human ovarian cancer, melanoma, pancreatic cancer and breast cancer high numbers of Tregs have been detected, which have been associated with a worse prognosis[17-20]. Tumors tend to secrete anti-inflammatory factors such as IL-10, TGF-β and Prostaglandine E2 (PGE2), creating an immunosuppressive milieu, which contributes to the in situ generation of Tregs. More specifically, tumor-infiltrating DCs will be polarized into tolerogenic DCs (tDCs) that induce Tregs both in tumor microenvironment as well as in the tumor-draining lymph nodes (TDLNs)[21,22]. In addition, tumors secrete (C-C motif) ligand 22 (CCL22) leading to the recruitment of nTregs into the tumor milieu[23].

We recently observed that sialylated antigens drive DCs to differentiate naive $CD4^+$ T cells into $CD4^+$ $FoxP3^+$ iTregs. However, it is unclear whether the uptake of sialylated antigens by DCs leads to similar effects on $CD8^+$ T cell differentiation. For this purpose, we modified Ovalbumin (OVA) with α2-6-linked Sias (Sia-OVA) and evaluated the effects of Sia-OVA- loaded DCs ($DC_{Sia\text{-}OVA}$) on $CD8^+$ T cell activation and differentiation. Our data reveal that $DC_{Sia\text{-}OVA}$ significantly arrested the proliferation of OVA-responsive $CD8^+$ T cells. In addition, we observed a reduced generation of GrB^+ and IFN-γ-producing $CD8^+$ effector T cells. Furthermore, $DC_{Sia\text{-}OVA}$ induced a distinct population of $CD8^+$ T cells expressing FoxP3 as well as high levels of CD39. Our data suggest that antigen modification

with Sias may be used as therapeutic treatment for disorders that are characterized by exacerbated $CD8^+$ T cell reactions.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories and used at 8-12 weeks of age. OT-I TCR transgenic mice[24] were bred and kept in our animal facility under specific pathogen-free conditions. All experiments were approved by the Animal Experiments Committee of the VUmc.

Cells

Bone marrow derived DCs were cultured as described with minor modifications[25]. Femur and tibia of C57BL/6 mice were removed, both ends were cut and the marrow was flushed with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, CA, USA). The resulting marrow suspension was passed over a 100 µm gauze to obtain a single cell suspension. After washing, $2x10^6$ cells were seeded per 100 mm dish (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) in 10 ml IMDM, supplemented with 10% FCS; 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (BioWhittaker, Walkersville, MD) and 50 µM β- mercaptoethanol (Merck, Damstadt, Germany) and containing 30 ng/ml recombinant murine GM-CSF (rmGM-CSF). At day 2, 10 ml medium containing 30 ng/ml rmGM-CSF was added. At day 5, another 30 ng/ml rmGM-CSF was added to each plate. From day 7 onwards, the non-adherent DCs were harvested and used for experiments. OVA-responsive $CD8^+$ T cells were isolated from spleen and lymph node single cell suspensions from OT-I tg mice using the mouse CD8 negative isolation kit (Invitrogen, CA, USA). The resulting $CD8^+$ T cell population was typically 90% pure as assessed by flow cytometry.

Modification of OVA with α2-6-Sias

Modification of OVA with α2-6-Sias was performed as previously described (Perdicchio et al., manuscript submitted). Presence of α2-6-Sias on Sia-OVA was detected by ELISA using the plant lecting Sambucus Nigra. Hereto, Sia-OVA was coated directly on NUNC Maxisorp plates (NUNC, Roskilde, Denmark), followed

by incubation with the biotinylated plant lectin Sambucus Nigra (Vector Labs, CA, USA) and peroxidase-labelled streptavidin (Sigma- Aldrich, St. Louis, MO; Fig. S1A). Alternatively, plates were incubated with OVA-specific monoclonal antibodies (10 μg/ml, Sigma-Aldrich, clone A6075), followed by peroxidase-labeled goat-anti-mouse antibodies to detect OVA (Fig. S1B)

$CD8^+$ T cell proliferation and differentiation

To assess proliferation of OVA-responsive $CD8^+$ T cells, cells were labelled with 5 mM 5,6- carboxylfluorescein succinimidyl ester (CFSE) and added to $2.5x10^4$ DCs that were previously loaded with Sia-OVA or native OVA (30 μg/ml). After 3 days, OT-I T cells were analyzed by flow cytometry. Alternatively, T cell proliferation was assessed by adding [^{3}H]-thymidine (1 μCi/well; Amersham Biosciences, NJ, USA) during the last 16 hr of a 72 hr culture and [^{3}H]- thymidine incorporation was assessed using a Wallac microbeta counter (Perkin-Elmer,USA).

To determine $CD8^+$ T cell differentiation, $5x10^4$ purified OT-I T cells were added to $1x10^4$ DCs, which were pulsed with 30 μg/ml Sia-OVA or native OVA 4 hr prior. To mimic T cell differentiation under inflammatory conditions, DCs were simultaneously incubated with antigens and 100 ng/ml LPS. After 2 days, 10 U/ml recombinant mouse IL-2 was added to the DC-T cell cultures. On day 7, T cells were analysed for expression of IFN-γ, GrB and FoxP3 by intracellular staining and flow cytometry. Consequently, cells were re-stimulated with 30 μg/ml PMA, 500 ng/ml ionomycin in the presence of 5 μg/ml Brefeldin A (Sigma-Aldrich). Five hours later, cells were permeabilized using the Cytofix/Cytoperm kit (BD biosciences, CA, USA) and incubated with anti-IFN-γ and anti-GrB antibodies for 30 min at 4°C. For FoxP3 analysis, cells were directly fixed and permeabilized using the specific FoxP3 buffer set (eBioscience, CA, USA) and incubated with anti-FoxP3 antibodies for 30 min at 4°C.

Antibodies

Antibodies were purchased from eBioscience and BD Biosciences. FITC-labelled antibodies used were anti-CD39 (418), anti-CD8b (H35-17.2), anti-MHC class I

(AF6-88.5), anti-PDL2 (122) and anti-Granzyme B (16G6). PE-labelled antibodies were anti-CD8b (eBioH35-17.2), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CD70 (FR70), anti-CD40 (1C10), anti-PD-L1 (MIH5), anti-CTLA-4 (DTA-1) and anti-GITR (UC10-4B9). APC-labelled antibodies were anti-FoxP3 (FJK-169) and anti-IFN-γ (XMG1.2).

Statistical analysis

Prism software (GraphPad 5.0) was used for statistical analysis. Student's t test, and Bonferroni multiple comparison tests were used to determine statistical significance. Statistical significance for all the tests, assessed by calculating the P values, was defined as $P<0.05$.

Results

Sia-OVA uptake by DCs inhibits DC-mediated $CD8^+$ T cell expansion and acquisition of effector function

To assess whether the $CD8^+$ T cell instructive abilities of DCs are modulated after uptake of sialylated antigens, we first evaluated the effects of sialylated antigen uptake on DC-mediated T cell expansion. Accordingly, BMDCs were loaded with $DC_{Sia\text{-}OVA}$ and co-cultured with OVA-specific $CD8^+$ OT-I T cells and after three days proliferation of $CD8^+$ T cells was evaluated by [^{3}H]-thymidine incorporation. This was compared with cultures containing BMDCs that were loaded with native OVA (DC_{OVA}) or none antigen pulsed control DCs (DC_{MED}). We observed that DC_{OVA} clearly promoted proliferation of OT-I T cells as indicated by high amounts of [^{3}H]-thymidine incorporation. By contrast, OT-I expanded significantly less when $DC_{Sia\text{-}OVA}$ were used (Fig. 1A). More specifically, $DC_{Sia\text{-}OVA}$-induced T cell proliferation was approximately 66% lower than the proliferation induced by DC_{OVA} (Fig. 1A). Even under inflammatory conditions, which were mimicked by adding Sia-OVA and the TLR4 ligand lipopolisaccharide (LPS) simultaneously to DCs, the lower T cell stimulatory capacity of $DC_{Sia\text{-}OVA}$ was retained (Fig. 1B). Under these conditions, T cell proliferation induced by $DC_{Sia\text{-}OVA}$ was 50% lower than by DC_{OVA}.

Labelling the OT-I T cells with CFSE enabled us to examine the effects of $DC_{Sia\text{-}OVA}$ on their proliferation in more detail. We observed that 56% of OT-I T cells that were co-cultured with DC_{OVA} had divided. The majority of OT-I T cells underwent 3 divisions and some of the cells underwent up to 6 divisions (Fig. 1C). By contrast, 98% of OT-I cells remained undivided upon co-culture with $DC_{Sia\text{-}OVA}$. In fact, this CFSE profile resembled that of OT-I T cells co- cultured with DC_{MED} (Fig. 1C). When DCs were loaded with OVA in the presence of LPS, the frequency of T cells that had expanded was increased. 84% of OT-I T cells had divided and the majority had undergone 5 divisions. In the presence of LPS, DCs that had taken up Sia- OVA did induce the expansion of OT-I T cells. However, in contrast to cultures containing DC_{OVA}/LPS, only 31% of the OT-I T cells were proliferating while the majority of the cells (69%) remained undivided (Fig. 1C). Control

DC_{MED} did not induce T cells to divide.

Given these findings on OT-I T cell proliferation, we next determined whether this is also reflected in the differentiation program of OT-I T cells. Hereto, OT-I T cells were analyzed after co-culture with $DC_{Sia\text{-}OVA}$ for production of IFN-γ and GrB, genuine markers of effector $CD8^+$ T cells. Of the $CD8^+$ T cells that were primed by DC_{OVA}, 23% produced IFN-γ (Fig.2A). By contrast, when activated by $DC_{Sia\text{-}OVA}$, less than 10% of the OT-I T-cells was able to produce IFN-γ (Fig. 2A). Similarly, $DC_{Sia\text{-}OVA}$ also minimized the numbers of GrB^+ OT-I T cells, as only 5% of GrB^+ OT-I T cells were detected compared to 11% GrB^+ OT-I T cells observed in DC_{OVA} containing cultures (Fig. 2B). In line with our observations on

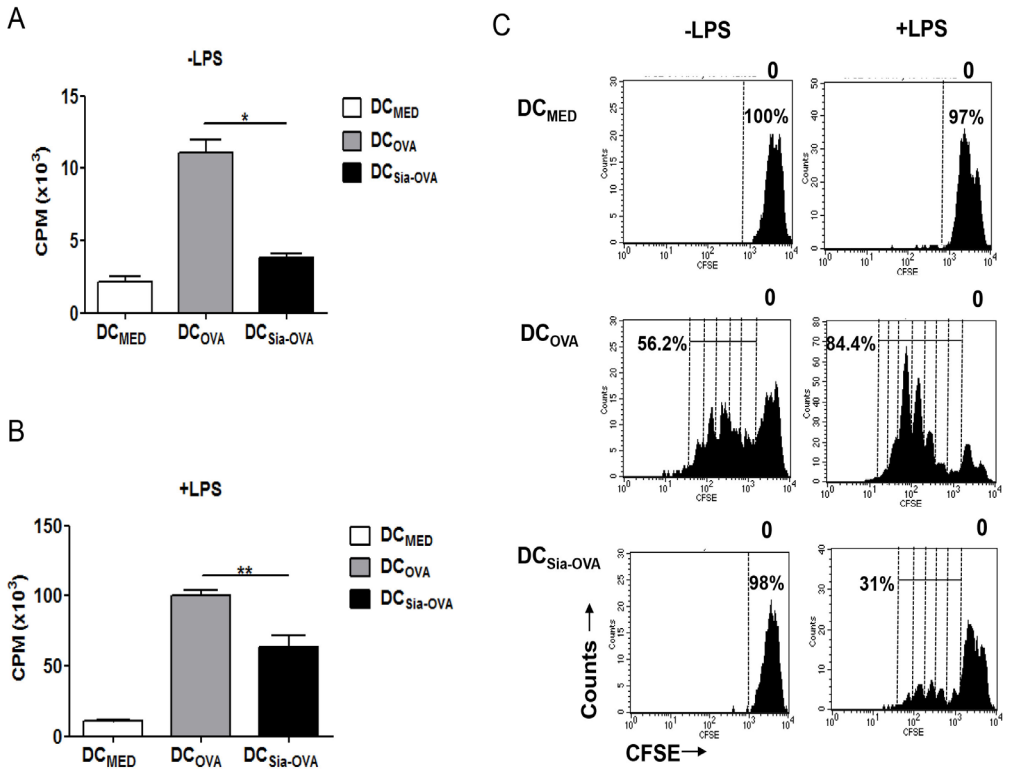

Figure 1. $DC_{Sia\text{-}OVA}$ restrain OVA-responsive $CD8^+$ T cell expansion. A+B, proliferation of OT-I T cells co-cultured with DC_{MED}, DC_{OVA}, or $DC_{Sia\text{-}OVA}$ with (A) or withouth (B) LPS as determined by [^{3}H]Thymidine icorporation (mean CPM ±s.e.m.). Data represent 4 independent experiments. *, P<0.05;**, p<0.01 (one-way Anova and Bonferroni multiple comparison tests). C, Proliferation of CFSE-labeled OT-I T cells as detected by fellow cytometry . Histograms were gated on live $CD8b^+$ T cells. Percentages of divided as well as undivided cells are indicated. Similar results were obtained in 3 independent experiments.

$CD8^+$ T cell proliferation, 50% less IFN-γ and GrB-producing OT-I T cells were generated by DCs that had taken up Sia-OVA in the presence of LPS than by DC_{OVA}/LPS (Fig. 2C).

Collectively, these data show that $DC_{Sia\text{-}OVA}$ limit OVA-responsive $CD8^+$ T cells to expand and gain effector functions. These effects were also apparent under inflammatory conditions, which suggest a strong inhibitory effect of Sia-OVA on the ability of DCs to initiate CTL responses.

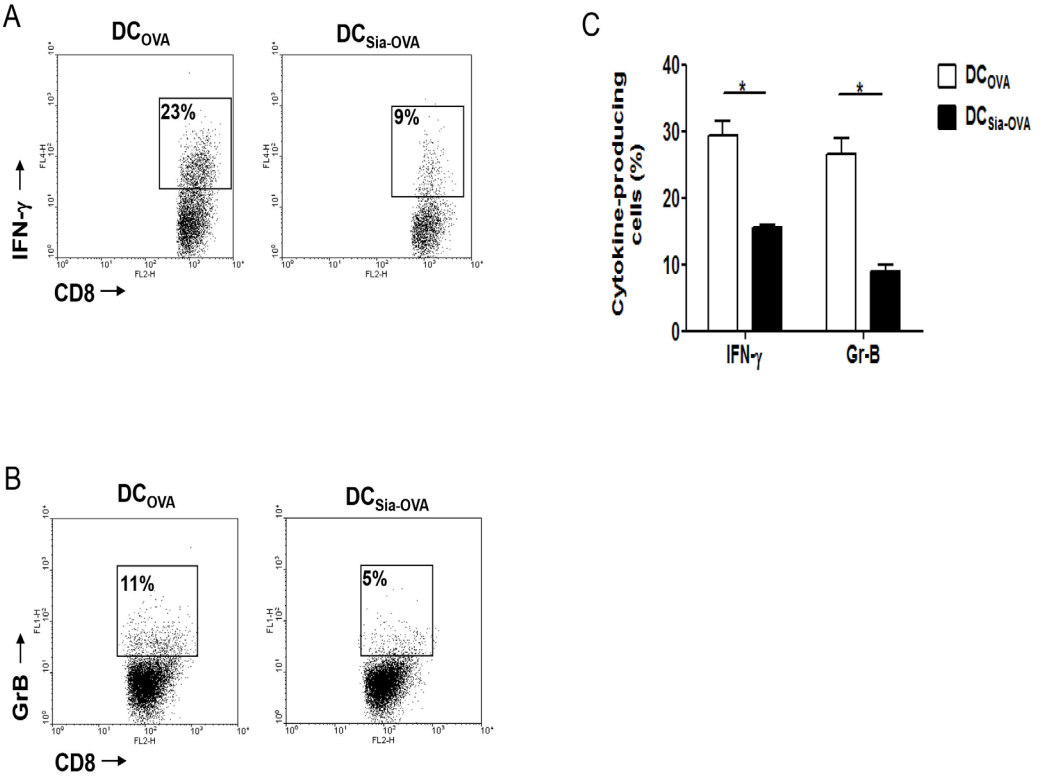

Figure 2. Reduced effector $CD8^+$ T cell generation by $DC_{Sia\text{-}OVA}$. A, analysis of IFN-γ^+ effector OT-I T cell frequencies after co-culture with DC_{OVA} or $DC_{Sia\text{-}OVA}$ as determined by flow cytometry. Plots were gated on live $CD8b^+$ T cells. Numbers in gates represent percentage of IFN-γ^+ of $CD8^+$ T cells. Data shown are representative of 3 independent experiments. B, Flow cytometric analysis of GrB^+ effector OT-I T cell frequencies after co-culture with DC_{OVA} or $DC_{Sia\text{-}OVA}$. Numbers in gates represent percentage of IFN-γ^+ of $CD8^+$ T cells. C, Frequency of effector OT-I T cells generated during co-culture with DCs that were incubated with Sia-OVA or OVA in the presence of LPS. Graphs represent the percentage of OT-I^+ IFN-γ^+ T cells and OT-I GrB^+ T cells (mean ±s.e.m.). *, $P<0.05$ (Student's t test).

Sia-OVA-loaded DCs promote CD8+ Tregs

The reduced proliferation of $CD8^+$ T cells as well as the impaired generation of effector T cells induced by $DC_{Sia\text{-}OVA}$ could reflect the conversion of $CD8^+$ in tolerogenic $CD8^+$ T cells ($CD8^+$ Tregs). To evaluate whether $DC_{Sia\text{-}OVA}$ can induce $CD8^+$ Tregs, OT-I T cells were co- cultured with either $DC_{Sia\text{-}OVA}$ or DCOVA and the expression of the Treg marker FoxP3 was assessed by flow cytometry in primed $CD8^+$ T cells. Notably, 7% of OT-I T cells activated by $DC_{Sia\text{-}OVA}$ expressed FoxP3. This population was absent in cultures containing DC_{OVA} (Fig. 3A). However, the simultaneous addition of Sia-OVA and LPS to DCs completely abolished the generation of the $CD8^+$ $FoxP3^+$ T cells population *in vitro* (Fig. 3B).

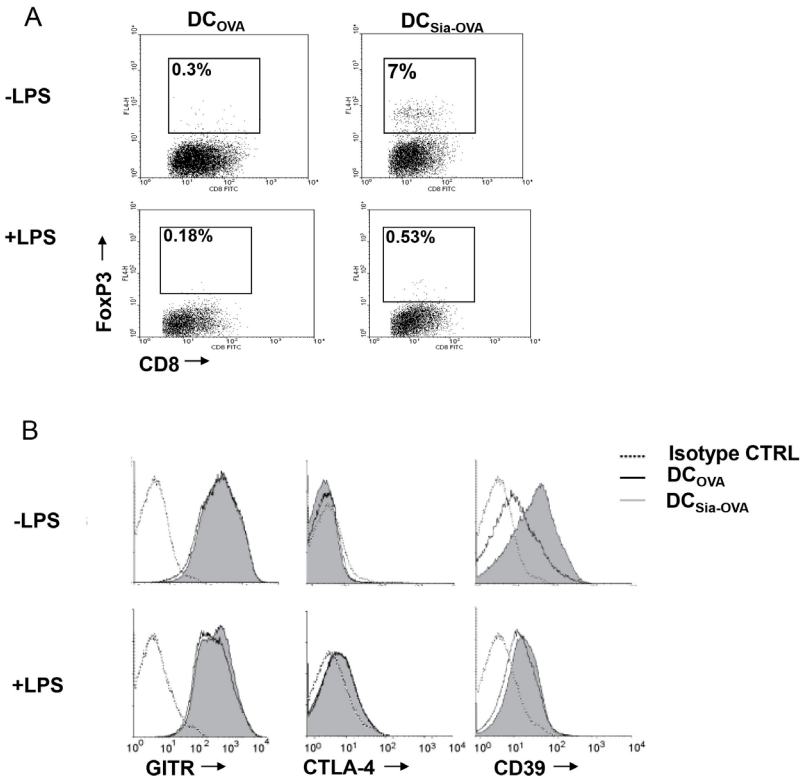

Figure 3. $DC_{Sia\text{-}OVA}$ promote induction of $CD8^+$ $FoxP3^+$ $CD39^{hi}$ T cells under homeostatic conditions. A+B, Expression of FoxP3 and IFN-γ expression in OT-I T cells after one week co-culture with DCs that were prior pulsed with Sia-OVA or OVA in the presence or absence of LPS as determined by flow cytometry. Plots were gated on $CD8b^+$ T cells. Numbers in gates indicate percentage of $CD8^+$ $FoxP3^+$ T cells. Shown is a representative example of 3 independent experiments. C, Expression of GITR, CTLA-4 and CD39 on OT-I T cells primed by $DC_{Sia\text{-}OVA}$ (full peack) or DC_{OVA} (black line) in the absence (upper panel) or presence (lower panel) of LPS. Dashed line represents isotype control staining.

Besides FoxP3, Tregs are often associated with high expression of other molecules such as GITR, CTLA-4 and CD39[26]. Therefore, we ascertained whether $DC_{Sia\text{-}OVA}$ induced the expression of these markers on primed OT-I T cells. Only $DC_{Sia\text{-}OVA}$ promoted high expression of CD39 ($CD39^{hi}$) on OT-I T cells (Fig. 3C). By contrast, equal expression levels of GITR were detected on T cells activated by $DC_{Sia\text{-}OVA}$ and DC_{OVA}, while CTLA-4 was neither expressed on DC_{OVA} nor on $DC_{Sia\text{-}OVA}$-primed OT-I T cells (Fig. 3C). We additionally analyzed the expression of these Treg-associated markers on OT-I T cells under inflammatory conditions. Likewise the expression of FoxP3, the addition of LPS abrogated the presence of $CD39^{hi}$ OT-I T cells in $DC_{Sia\text{-}OVA}$ primed OT-I T cell cultures (Fig. 3C), indicating that under inflammatory conditions induction of $CD8^{+}$ $FoxP3^{+}$ Tregs by $DC_{Sia\text{-}OVA}$ does not occur while the capacity to inhibit effector T cell generation is maintained.

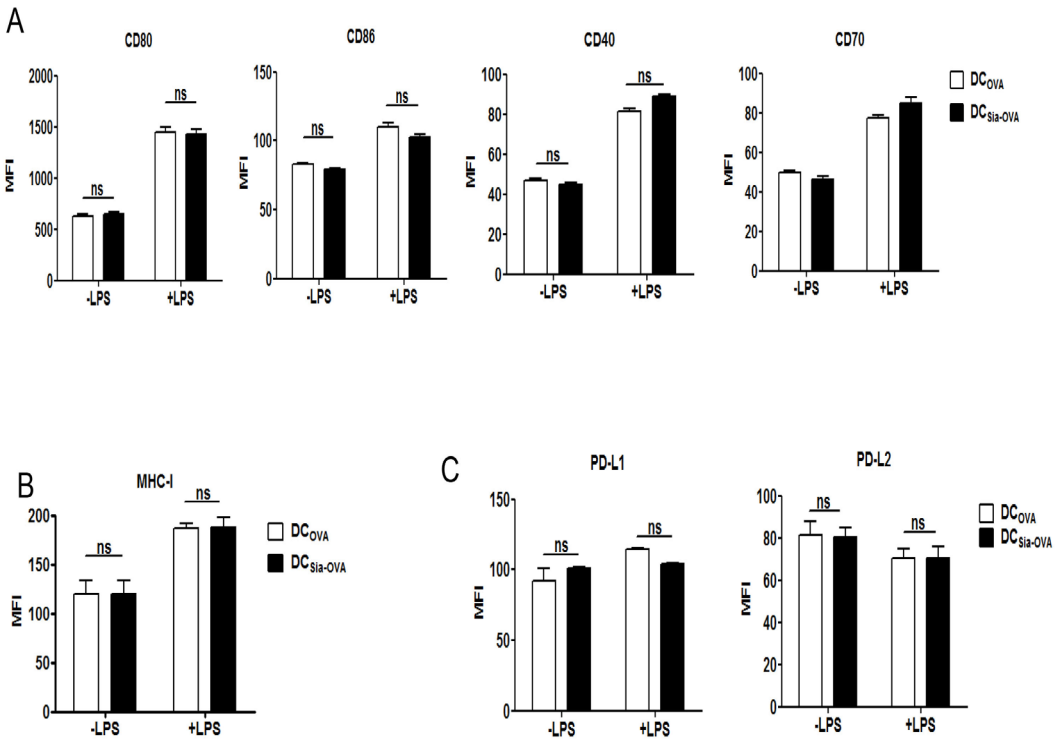

Figure 4. The uptake of Sia-OVA by DCs does not change DC phenotype. A+B+C, Expression analysis of CD80, CD86, CD70 and CD40 (A), MHC-I (B), PD-L1 and PD-L2 (C) on DCs following Sia-OVA or unmodified OVA uptake by flow cytometry. Graphs represent the Mean Fluorescent Intensity (MFI) of molecule expression (mean ± s.e.m.). Data represent 5 independent experiments. Ns, not significant (Student's t test).

Sia-OVA uptake by DCs may lead to reduced antigen presentation due to reduced expression of MHC I molecules and/or altered co-stimulatory molecule expression, thereby promoting OT-I Tregs. We therefore assessed the expression of these molecules on DCOVA or $DC_{Sia\text{-}OVA}$ in the absence and presence of LPS. $DC_{Sia\text{-}OVA}$ did not exhibit a different signature compared to DC_{OVA} in terms of expression levels of CD80, CD86, CD70 and CD40 (Fig. 4 A) as well as MHC class I (Fig. 4 B). The induction of OT-I Tregs by DCSia-OVA could result from instructive signals by tolerogenic molecules such as program cell death ligand-1 and -2 (PD-L1/PD-L2)[27]. However, our analysis revealed similar expression of these tolerogenic markers on DC_{OVA} and $DC_{Sia\text{-}OVA}$ (Fig. 4 C). In the presence of LPS, expression of costimulatory and MHC class I molecules was enhanced, yet remained equal between $DC_{Sia\text{-}OVA}$ and DC_{OVA}. Moreover, in the presence of LPS, IL-10 and TGF-β mRNA levels were equal between DC_{OVA} and $DC_{Sia\text{-}OVA}$ (Fig. 5A+B). Thus, until now we did not unveil a specific tolerogenic (surface) marker on $DC_{Sia\text{-}OVA}$.

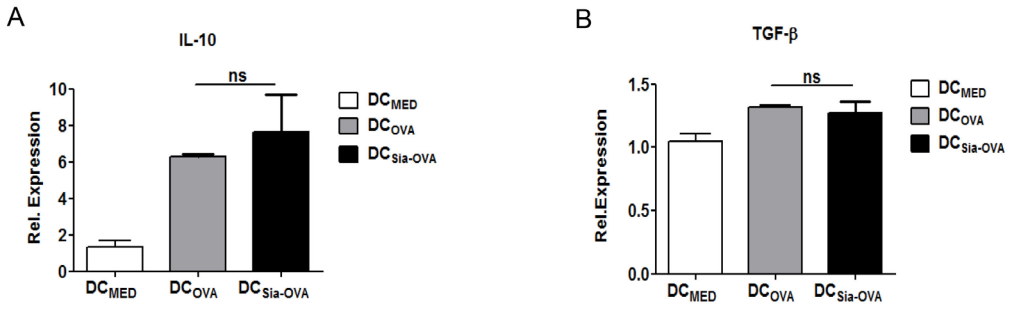

Figure 5. DCOVA and $DC_{Sia\text{-}OVA}$ present equal expression of anti-inflammatory cytokines in the presence of LPS. A+B, gene expression of IL-10 (A) and TGF-β (B) secreted by DC_{OVA}, $DC_{Sia\text{-}OVA}$, or unpulsed DCs (DC_{MED}) as determined by Real-time PCR. Graphs indicate the relative expression of GAPDH (mean ± s.e.m.). ns, not significant (Student's t test).

Discussion

Here, we show that DCs loaded with Sia-modified OVA strongly suppress the expansion of OVA-responsive $CD8^+$ T cell and significantly restrain acquisition of cytotoxic effector functions as determined by reduced numbers of GrB- and IFN-γ-producing T cells. Moreover, we observed that a proportion of the $CD8^+$ T cells activated by Sia-OVA-loaded DCs expressed the Treg-associated markers FoxP3 and CD39. Induction of tolerogenic $CD8^+$ T cells was abrogated when Sia-OVA was taken up in the presence of LPS, yet the formation of cytotoxic effector $CD8^+$ T cells was still restrained by these DCs.

Our findings indicate that the modification of OVA with Sias significantly suppressed the capacity of DCs to induce the outgrowth as well as the differentiation of OVA-responsive $CD8^+$ T cells into CTLs. CTLs play crucial functions in the immune response against viral infections. However, certain viruses such as Herpes simplex virus (HSV)-1 and -2 adopt latency as strategy to evade immune defense and indefinitely persist in the host. Infections caused by these viruses are characterized by low antigen expression and low level of $CD8^+$ T cell activation[28]. Since high levels of Sias have been found on the envelope glycoproteins of HSV-1/-2[29], it can be hypothesized that Sias on HSV contribute to HSV immune escape by tolerizing DCs.

We have recently shown that modification of antigens with Sias induced tolerogenic DCs that promoted de novo generation of $CD4^+$ Tregs via DCs. Our current studies show that $DC_{Sia\text{-}OVA}$ also induced the generation of a $CD8^+$ T cell population that expresses FoxP3 and CD39. CD39 is an ectonucleotidase involved in the degradation of extracellular ATP, inhibiting the pro-inflammatory functions of innate immune cells and their capacity to initiate a Th1 response. So far, CD39 expression has been prevalently detected on $CD4^+$ $CD25^{hi}$ and $CD4^+$ $FoxP3^+$ Tregs, in particular on $CD4^+$ Tregs of patients with human T-lymphotropic virus type 1 (HTLV), human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections[30-32]. $CD8^+$ $CD39^{hi}$ expressing T cells have been detected in patients with renal, bladder or colorectal cancer in which they are involved in the suppression of effector $CD8^+$ T cell activities[33]. The increased expression of CD39 on $CD8^+$ T cells that were primed by $DC_{Sia\text{-}OVA}$ may thus suggest the

generation of a $CD8^+$ $FoxP3^+$ Treg population that exerts its immunosuppressive functions through metabolic disturbance rather than inhibit immune functions in a cell-contact dependent mechanism involving CTLA-4, which was absent on these cells.

Both $CD4^+$ and $CD8^+$ induced and natural occurring Tregs have the pivotal role of maintaining immune homeostasis in order to prevent excessive and unwanted immune reactions that can lead to autoimmune and inflammatory diseases[34]. In contrast to $CD4^+$ Tregs, much less is known about $CD8^+$ Tregs, especially because of lacking of potential markers that can better characterize them. The well defined $CD8^+$ Tregs are the thymus-derived $CD8^+$ $CD44^+$ $ICOSL^+$ Tregs that suppress functions of T cells in a manner involving the non- classical MHC class 1b (Qa-1)[35]. However, other subsets of natural or induced $CD8^+$ Tregs have emerged. These include $CD8^+$ $CD28^-$ Tregs, which are associated with the pathogenesis of Reumatoid Arthritis (RA) and systemic lupus erythematosus (SLE)[36 37]. $CD8^+$ $CD28^-$ $FoxP3^+$ subsets have been found to induce allogeneic tolerance in mice through the suppression of $CD4^+$ T cell functions by inducing the down-regulation of co-stimulatory molecules such as CD80, CD86 as well as the up-regulation of inhibitory receptors immunoglobulin transcript (ILT)-3 and -4 on DCs[38]. Our findings thus may indicate that $DC_{Sia-OVA}$ induce a $CD8^+$ $FoxP3^+$ T cell population with tolerogenic and immunosuppressive features.

Intriguingly, dampening of effector T cell generation seems to occur via a different process than induction of $FoxP3^+$ $CD8^+$ Treg. Our findings demonstrated that co-addition of Sia-OVA and LPS to DCs inhibited the generation of $CD8^+$ Tregs as we did not detect $FoxP3^+$ and $CD39^{hi}$ $CD8^+$ T cells. However, under these conditions, $DC_{Sia-OVA}$ still impaired CTL differentiation. The expression of MHC-I on $DC_{Sia-OVA}$ was equal to that on DC_{OVA}, excluding thus the possibility that the inhibition of effector cells and/or induction of $CD8^+$ Tregs are a consequence of reduced antigen-presentation[39]. Moreover, $DC_{Sia-OVA}$ did not show tolerogenic signatures compared to DC_{OVA} as they express similar expression levels of the co-stimulatory molecules CD80, CD86, CD70, CD40 and the tolerogenic molecules PD-L1 and PD-L2.

Previous studies revealed that tolerogenic effects of $DC_{Sia-OVA}$ are mediated via a

cell contact- dependent fashion (Perdicchio et al., manuscript submitted), which could include the involvement of ILT-3 annd ILT-4, as high expression of these molecules on DCs has been connected with $CD8^+$ Treg induction. However, the involvement of ILT-3 and -4 in the tolerogenic effects of $DC_{Sia\text{-}OVA}$ needs to be evaluated in future studies.

In conclusion, our findings introduce a potential beneficial use of sialylated antigens to impair and dampen unwanted $CD8^+$ T cell responses. Exacerbated $CD8^+$ T cell reactions contribute to the pathogenesis of auto-immune diseases such as Type 1 Diabetes[40]. Hence, modification of disease-related-antigens with sialic acids might dampen antigen-specific $CD8^+$ cytotoxic T cells and especially promote antigen $CD8^+$ Tregs that may recover the disease.

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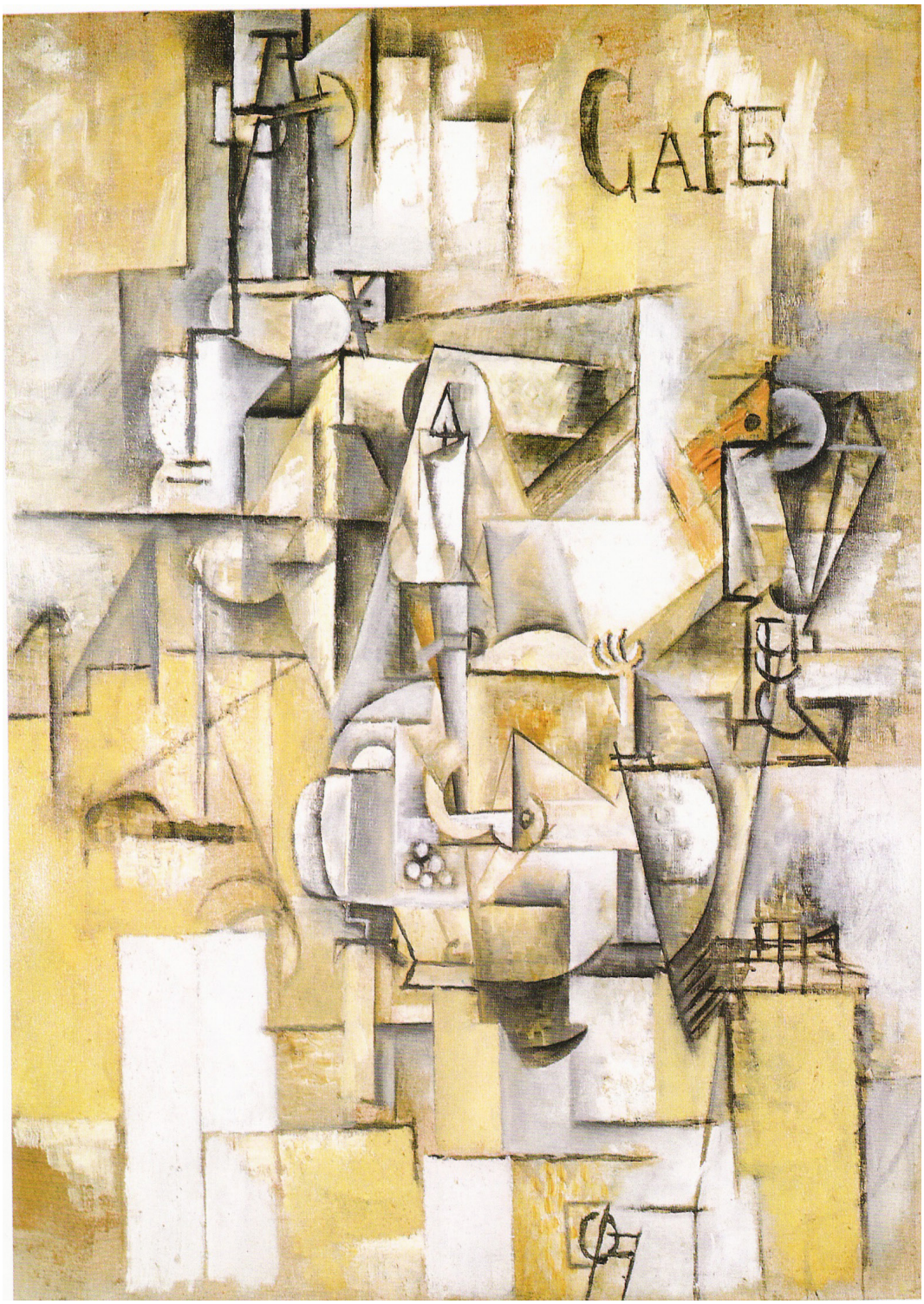

above: Pablo Picasso; *Le pigeon aux petits pois* (1911).

Chapter 5

Characterization of dendritic cells loaded with sialic acid-modified antigens

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Manuscript in preparation

Abstract

In our previous studies we showed that the presence of sialic acids (Sias) on antigen modulated the capacity of Dendritic Cells (DCs) to initiate $CD4^+$ and $CD8^+$ T cell-mediated immune responses promoting immunological tolerance rather than effector immunity. However, mechanisms through which Sia-antigen-loaded DCs divert T cell activation are not understood. Here we examined whether the modulation of T cell activation by Sia-antigen- loaded DCs occurs in a cytokine-dependent or in a cell-contact-dependent manner. We observed that culturing $CD4^+$ T cells with DCs that received Sia-modified OVA ($DC_{Sia\text{-}OVA}$) or unmodified OVA (DC_{OVA}) significantly impaired IFN-γ^+ $CD4^+$ T cell differentiation. In contrast, when DCs and T cells were separated using a transwell system, the T cell inhibitory effects were not present. The observation of a cell-contact dependent T cell differentiation induced by $DC_{Sia\text{-}OVA}$ led us to study the altered gene expression profile of $DC_{Sia\text{-}OVA}$. We found that after 1 hour of contact with Sia-OVA antigen, DCs displayed a clear up-regulation of genes encoding Sia-binding Siglec-E as well as tolerogenic DC-associated molecules such as Immunoglobulin-like transcript (ILT)-3. In addition, Sia-OVA induced the up-regulation of genes involved in the mammalian target of rapamycin (mTOR) pathway, which has been implicated in the regulation of DC activation. In conclusion, we here dissected the involvement of several markers in DCs that may be involved in the inhibitory modulation of T cell function, and we identified candidate genes that may be involved in the regulatory effects that sialylated antigen imposes on DCs.

Introduction

T cell-mediated immunity represents a valid protection against pathogens and tumor cells that succesfully evade the innate immune system. The initiation of a T cell response involves the presentation of pathogen or tumor antigens by Dendritic Cells (DCs) in the context of major histocompatibility complex (MHC) class-I to initiate a $CD8^+$ T cell response, or in the context of MHC class-II for $CD4^+$ T cell activation. However, to fully initiate T cell responses, DCs provide other signals that are represented by the up-regulation of co-stimulatory molecules and the production of cytokines in response to danger perception via pattern recognition receptors (PRRs)[1]. All together, these signals provided by DCs ensure the differentiation of $CD4^+$ T cells into a specific and appropriate T helper subset as well as induction of cytotoxic $CD8^+$ T cells (CTLs) that potently kill virally infected or tumor cells.

In our previous studies, we showed that the modification of antigens with sialic acids (Sias) affect the ability of DCs to elicit the effector T cell response. In particular, targeting of DCs with different linked sialylated lipooligosaccharide structures of Campylobacter jejuni determine the differentiation of human $CD4^+$ T cells into T helper 1 (Th1) and T helper 2 (Th2)[2]. Furthermore, we have also demonstrated that DCs receiving Ovalbumin that was modified with Sias ($DC_{Sia-OVA}$) promoted the *de novo* generation of $CD4^+$ regulatory T cells (Tregs) at the expense of effector $CD4^+$ T cells in vitro and in vivo (Perdicchio et al., manuscript submitted). Notably, the $CD8^+$ T cell activation program was also altered by $DC_{Sia-OVA}$, which potently suppressed the activation of CTLs, inducing $CD8^+$ Tregs instead (Perdicchio et al., manuscript in preparation). However, the mechanisms how uptake of sialylated antigens affects the capacity of DCs to activate and differentiate T cells are not completely understood.

Here we investigated the process how DCs encountering Sia-OVA induce Treg responses. Using transwell experiments, we excluded the involvement of a soluble factor in Sia-mediated effects imposed on DCs. By gene expression profiling we characterized potential candidates that are involved in the induced regulatory phenotype of $DC_{Sia-OVA}$ or lead to tolerogenic T cell responses in a cell-contact dependent mechanism. We observed that $DC_{Sia-OVA}$ showed the up-regulation of Sia-

binding lectin (Siglec)-E. Moreover, we detected increased expression of Immunoglobulin-like transcript (ILT)-3 and the mammalian target of rapamycin (mTOR) pathway, both previously associated with DCs with tolerogenic function.

Materials and Methods

Cells

Bone marrow derived Dendritic Cells (BMDCs) were cultured as described by Lutz et al. with minor modifications[3]. Femur and tibia of C57BL/6 mice were removed, both ends were cut and the marrow was flushed with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, CA, USA). The resulting bone marrow suspension was passed over 100 μm gauze to obtain a single cell suspension. After washing, $2x10^6$ cells were seeded per 100 mm dish (Greiner Bio- One, Alphen aan de Rijn, The Netherlands) in 10 ml IMDM, supplemented with 10% FCS; 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (BioWhittaker, Walkersville, MD) and 50 μM β-mercaptoethanol (Merck, Damstadt, Germany) and containing 30 ng/ml recombinant murine GM-CSF (rmGM-CSF). At day 2, 10 ml medium containing 30 ng/ml rmGM-CSF was added. At day 5, another 30 ng/ml rmGM-CSF was added to each plate. From day 7 onwards, the non-adherent DCs were harvested and used for subsequent experiments.

T cell differentiation

For analysis of $CD4^+$ T cell differentiation, $1x10^4$ DCs were pulsed with 30 μg/ml Sia-OVA or native OVA for 4 hours in round bottom 96-wells plates. Subsequently, antigen-pulsed DCs were washed and $5x10^4$ purified naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ OT-II T cells were added to each well. After 2 days, 10 U/ml recombinant mouse IL-2 was added. On day 6, polarization of T cells was analysed. Hereto, cells were re-stimulated with 30 μg/ml PMA, 500 ng/ml Ionomycin in the presence of 5 μg/ml Brefeldin A (Sigma), and 5 hours later the expression of FoxP3 and IFN-γ was analysed by flow cytometry. In brief, T cells were stained for surface markers for 30 min at 4°C, fixed and permeabilized with the FoxP3 Staining Buffer Set (eBioscience, San Diego, CA, USA) O/N at 4°C and subsequently incubated with FoxP3 or IFN−γ specific antibodies for 30 min at 4°C. To determine whether $DC_{Sia\text{-}OVA}$ induce Treg differentiation via a soluble factor, cells were cultured in Transwell plates with a 0.4 μM pore size

(Millipore, Billercia, MA). Antigen-pulsed DCs and purified naive $CD4^+$ $CD62L^{hi}$ $CD25^-$ OT-II were co-cultured in the lower chamber, while Sia-antigen-pulsed DCs were plated in the upper chamber. After 2 days, 10 U/ml recombinant mouse IL-2 was added to the lower chamber, and on day 7 T cells were analysed for the expression of IFN-γ and FoxP3 as described above.

RNA preparation, processing and microarray hybridization

One hour after the stimulation of DCs with Sia-OVA or OVA, RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel, Germany). RNA purity and concentration was assessed by Nanodrop (ThermoScientific). Pure RNA had an A260/A280>2. RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent technologies). RNA integrity and microarray hybridization were performed at ServiceXS, Leiden, The Netherlands.

Statistical analysis

Prism software (GraphPad 5.0) was used for statistical analysis. Student's t test was used to determine statistical significance. Statistical significance for all the tests, assessed by calculating the P values, was defined as $P<0.05$.

Results and discussion

Previously, we showed that DCs loaded with sialylated antigen promoted Treg induction and prevented the generation of effector T cells. In particular, when co-cultured with OVA-responsive $CD4^+$ T cells, $DC_{Sia\text{-}OVA}$ induced 20% of T cells to express FoxP3 and, after co-culture with OVA-responsive $CD8^+$ OT-I T cells, 7% of OT-I cells became $FoxP3^+$ (Fig.1 A) as already shown in chapter 3 (Fig. 1A) and chapter 4 (Fig. 3A). In addition, $DC_{Sia\text{-}OVA}$ potently impaired the differentiation of OT-I and OT-II T cells into effector IFN-γ-producing T cells (Fig. 1B). These effects were also observed in vivo suggesting thus a strong effect of sialylated antigen in the induction of immune tolerance and prevention of effector immunity (Perdicchio et al., manuscript submitted). However, till now it has not been clear how, upon uptake by DCs, sialylated antigens alter the DC phenotype and function into a regulatory one that leads to the dampening of T cell responses.

Analyzing the phenotype of DCs upon incubation with Sia-OVA or OVA antigen, we have not observed any differential expression of co-stimulatory molecules that mediate potent priming of $CD4^+$ and/or $CD8^+$ T cells such as CD80, CD86, CD40 and CD70 between $DC_{Sia\text{-}OVA}$ and DC_{OVA}. Moreover, $DC_{Sia\text{-}OVA}$ did not present an altered expression of tolerogenic molecules such as program cell death ligand (PD-L)1 and 2, whose overexpression on DCs has been associated with tolerogenic DCs (tDCs). Sia-OVA uptake by DCs did not alter the expression of MHC-I and MHC-II molecules, excluding thus the possibility that the tolerogenic effects of $DC_{Sia\text{-}OVA}$ are a consequence of a reduced antigen-presentation (Perdicchio et al., manuscript submitted). Furthermore, $DC_{Sia\text{-}OVA}$ did not secrete higher levels of anti-inflammatory cytokines such as IL-10 than DC_{OVA}. Thus, it is unclear how the uptake of Sia-OVA can modify the function of DCs to potently instruct a regulatory function in T cells.

To elucidate mechanisms leading to Tregs and impairment of effector T cell generation, we started to assess whether $DC_{Sia\text{-}OVA}$ change $CD4^+$ T cell activation in a manner that does not involve direct contact between DCs and T cells. More specifically, DCs were pulsed with Sia- OVA for 4 hours and transferred to the top well of a transwell plate containing the co-culture of DC_{OVA} and naive OT-II T

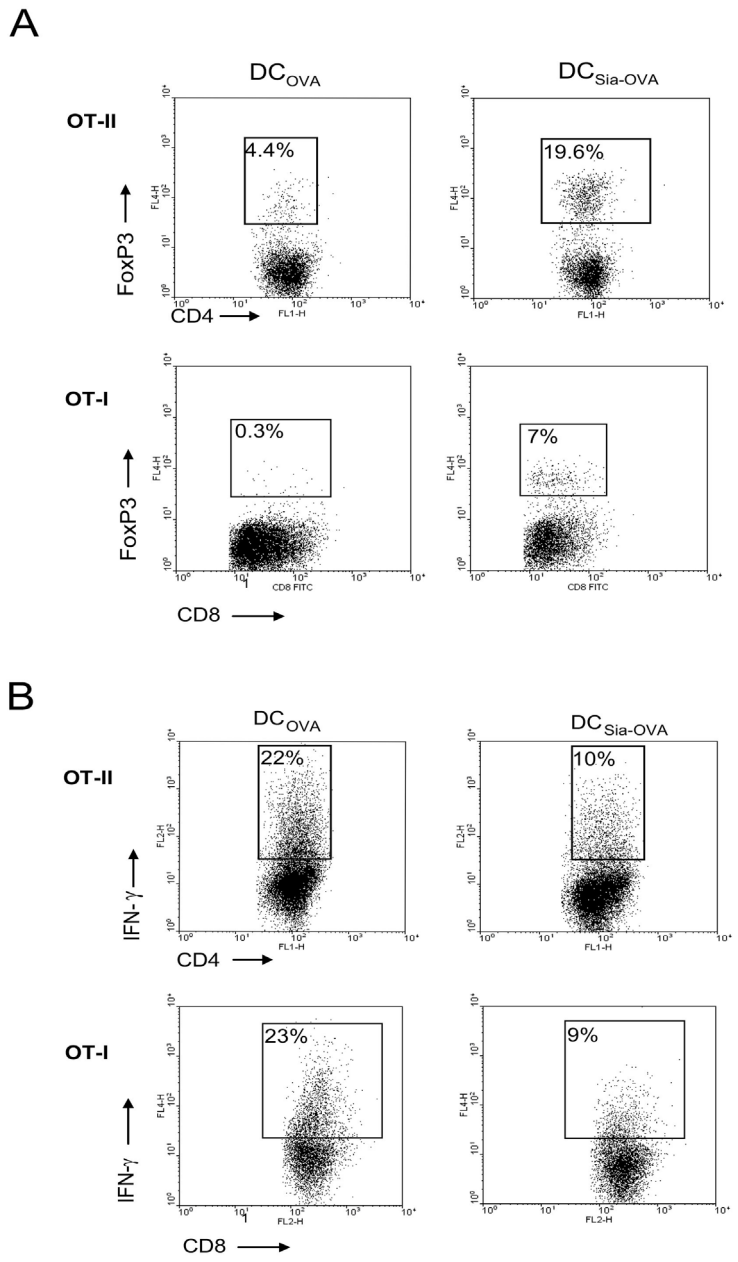

Figure 1. DCs that have taken up Sia-OVA induce the generation of CD4$^+$ and CD8$^+$ Treg and simultaneously prevent CD4$^+$ and CD8$^+$ effector T cell generation. A, Flow cytometric analysis of CD4$^+$ FoxP3$^+$ and CD8$^+$ FoxP3$^+$ T cells after co-culture of naive OT-II or OT-I T cells with DC$_{OVA}$ or DC$_{Sia-OVA}$. Dot plots are gated on CD4$^+$ or CD8$^+$ T cells. Numbers in gates indicate percentage of FoxP3$^+$ T cells. Dot plots are representative of five independent experiments. B, flow cytometry of IFN-γ$^+$ CD4$^+$ T cells and IFN-γ$^+$ CD8$^+$ T cells after co-culture of naive OT-II or OT-I T cells with DC$_{OVA}$ or DC$_{Sia-OVA}$. Plots are gated on CD4$^+$ or CD8$^+$ T cells. Percentage of IFN-γ$^+$ T cells are indicated. Shown is a representative of two independent experiments.

cells at the bottom. As a control, $DC_{Sia\text{-}OVA}$ and $CD4^+$ T cells were not separated by a membrane and cultured in the same well together. Upon examining IFN-γ-producing OT-II T cells, we observed that the generation of IFN-γ^+ OT-II T cells was not influenced by the presence of $DC_{Sia\text{-}OVA}$ in the top well of the transwell plate (Fig. 2A). Notably, we clearly detected a reduced percentage of primed IFN-γ^+ OT-II T cells when $DC_{Sia\text{-}OVA}$ were mixed with $CD4^+$ T cells, indicating that the effects of $DC_{Sia\text{-}OVA}$ on T cell occurred only via direct contact with $CD4^+$ T cells.

Given the observation of a cell contact-dependent inhibition of effector $CD4^+$ T cell differentiation and Treg induction by $DC_{Sia\text{-}OVA}$, we next analyzed the gene expression profile of DCs upon Sia-OVA uptake and concentrated on identifying cell surface molecules that could be involved in tolerance induction. Specifically, DCs were pulsed with either Sia-OVA or OVA, and 1 hour later RNA was isolated and processed for gene microarray analysis. Comparison of $DC_{Sia\text{-}OVA}$

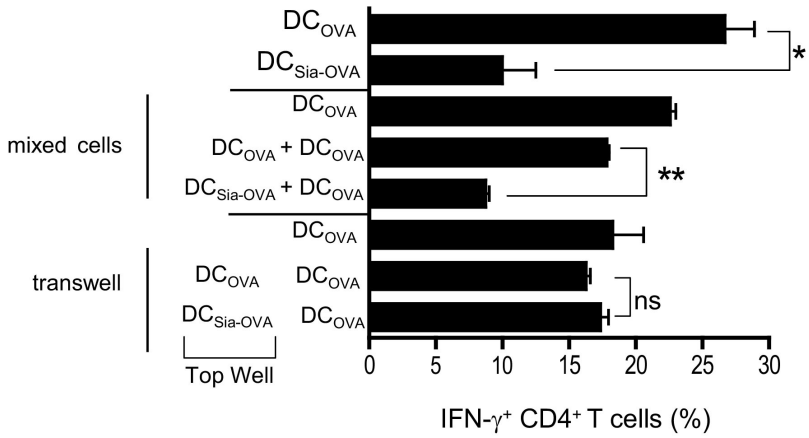

Figure 2. $DC_{Sia\text{-}OVA}$ dampen effector T cell generation in a cell-contact manner. $DC_{Sia\text{-}OVA}$ (top well) were separated from DC_{OVA} and naive OT-II T cells (bottom well) in a transwell plate. As control $DC_{Sia\text{-}OVA}$ and naive OT-II T cells were cultured together. On day 6, the OT- II T cells were analyzed by flow cytometry for IFN-γ expression. Graph indicates the % of IFN-γ^+ $CD4^+$ T cells (mean ± s.e.m.). *, $P<0.05$; ns, not significant (Student's t test).

and DC_{OVA} revealed several differentially expressed genes that are particularly interesting for our investigations. In particular, $DC_{Sia\text{-}OVA}$ displayed approximately 100-fold higher expression of Sialic acid binding receptor *Siglec-E* than DC_{OVA} (Table I). Siglec-E is a CD33-related Siglec mainly expressed on DCs, monocytes and neutrophils[4]. Interaction of Siglec-E with Sias results in tyrosine phosporylation of the intracellular ITIM domain by Src-family kinases followed by the recruitment of Src homology 2 domain-containing inositol polyphosphate 5' phospatases (SHIPs) or Src homology 2 domain-containing protein phosphatases (SHPs)[5,6]. These phosphatases lead to inhibitor signaling pathways that increase the activation threshold of the innate immune cells. Accordingly, expression profiling of $DC_{Sia\text{-}OVA}$ showed the overexpression of *INPP5D* gene, which encodes SHIP-1 phosphatase (Table I). However, further investigations are required to exactly identify the downstream pathway occurring in $DC_{Sia\text{-}OVA}$ leading to the tolerogenic phenotype.

Besides their role in the modulation of cell functions, CD33-related Siglecs are implicated in the internalization of sialylated antigens, leading subsequently to antigen processing and presentation for T cell activation[7]. In our previous studies, we showed that the absence of Siglec-E on DCs significantly reduced binding and uptake of Sia-OVA and totally arrested the DCSia-OVA-mediated generation of $CD4^+$ Tregs (Perdicchio et al., manuscript submitted). Therefore, the identification of Siglec-E mRNA overexpression in $DC_{Sia\text{-}OVA}$ enhanced the crucial role of this Siglec in mediating the tolerogenic effects of $DC_{Sia\text{-}OVA}$ T cell activation. $DC_{Sia\text{-}OVA}$ presented an increased expression of the ILT-3 receptor, whose expression has been associated with tDCs[8] (Table I). ILT-3 together with ILT-4 and ILT-2 are mainly expressed on DCs[9]. ILT-3 is up-regulated on DCs in response to immunosuppressive signaling such as IL-10, vitamin D and immunosuppressive $CD8^+$ T cells. ILT-3-overexpressing DCs show reduced expression of co-stimulatory molecules and increased production of IL-10 and TGF-β, leading to anergic T cells and Treg induction[10-13]. However, we have previously demonstrated that the uptake of Sia-OVA by DCs did not alter the expression of co-stimulatory molecules such as CD80 and CD86 or secretion of anti-inflammatory cytokines such as IL-10 compared to OVA uptake (Perdicchio et al., manuscript submitted). Therefore, according to the gene expression profile, it is possible that

$DC_{Sia\text{-}OVA}$ induce Tregs and prevent effector T cell generation in a ILT-3-independent manner or in a manner not involving the down-regulation of co-stimulatory molecules and the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. However, further studies are needed to evaluate whether also modification of $CD8^+$ T cell activation by $DC_{Sia\text{-}OVA}$ occurs in a cell contact-dependent fashion.

Interestingly, analysis of the gene expression profile of DC_{SiaOVA} also unveiled the upregulation of members of the mammalian target of rapamycin (mTOR) pathway (Table I). mTOR is a key signaling serine\threonine kinase involved in different aspects of cellular functions such as cell metabolism, growth and survival[14]. During immunity, mTOR plays an important role in the activation of T cell responses[15]. Upon T-cell receptor (TCR) and CD28 stimulation, mTOR

Table I. Differentially regulated genes in $DC_{Sia\text{-}OVA}$ compared to DC_{OVA}.

Gene name	Alias	Fold higher in $DC_{Sia\text{-}OVA}$
SIGLECE	Sialic acid binding Ig-like lectin E	102
SHIP-1	inositol polyphosphate-5-phosphatase	9
LILRB4 (ILT3)	Leukocyte immunoglobulin-like receptor subfamily B member 4	10.2
TSC1	Tuberous sclerosis 1 protein (mTOR pathway)	10.43
TSC2	Tuberous sclerosis 2 protein (mTOR pathway)	101.3669
PIK3c2a	Phatidylinositide 3-kinase, class 2, alpha polypeptide (mTOR pathway)	10
4E-BP1	eukaryotic translation initiation factor 4E binding protein 1 (mTOR pathway)	9.855
p70S6K	ribosomal protein S6 kinase, 70kDa, polypeptide 1 (mTOR pathway)	10.02
Akt1s1	AKT1 substrate 1 (mTOR pathway)	10.22

mediates T cell proliferation and promotes their survival. In addition, it has been observed that the specific mTOR inhibitor rapamycin not only inhibited these processes but also promoted the expansion of Tregs. In contrast to its role in T cells, the effects of mTOR signaling in DCs are still unclear. In myeloid DCs, the mTOR pathway modulates the secretion of pro-inflammatory cytokines IL-6, IL-12 and TNF-α in response to pathogen infection. It was shown that inhibition of mTOR using rapamycin increased the secretion of these pro-inflammatory cytokines. In addition, using murine BMDCs it was demonstrated that the effect of rapamycin is mediated by enhancing NF-KB and STAT3 activity[16-18]. These studies indicate that mTOR acts as a negative feedback regulator of activation in these DCs.

The analysis of $DC_{Sia\text{-}OVA}$ gene expression gave us new insights about the molecules potentially involved in the tolerogenic effects of $DC_{Sia\text{-}OVA}$. In particular, it would be interesting to investigate whether mTOR activity is enhanced in DCs upon the uptake of Sia- OVA.

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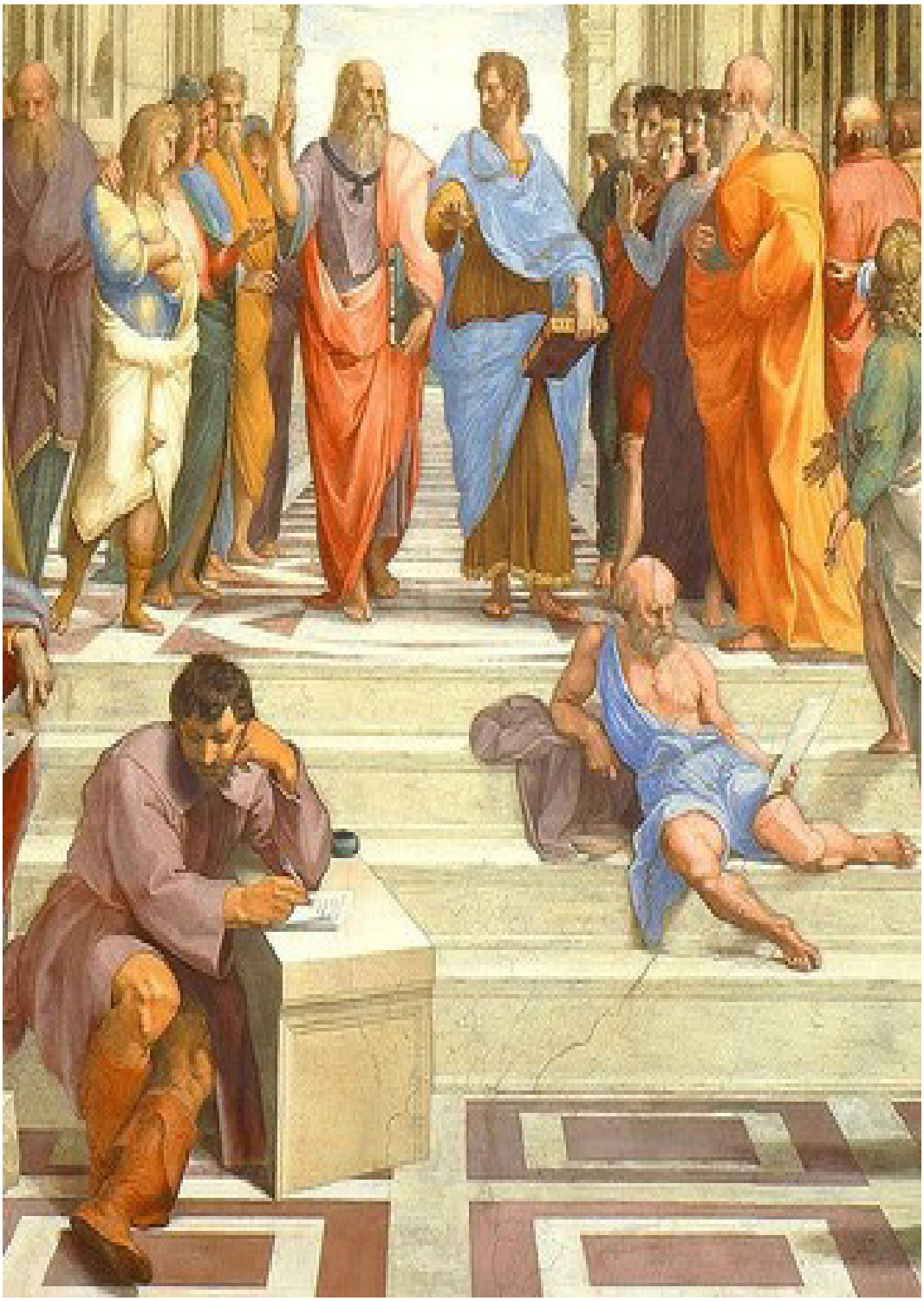


above: Raphael; detail of *The School of Athens* (1509-10). Apostolic Palace, Vatican City.

Chapter 6

General Discussion

The immune system provides us protection from diseases caused by invading bacteria, viruses, fungi, parasites and transformed cells. The innate and acquired immune system cooperate to eliminate the evading pathogen or the transformed cells, while maintaining homeostasis in absence of danger. In particular, the immune system provides immune tolerance towards self-antigens and harmless exogenous antigens to prevent an excessive and unwarranted immune reaction that can be harmful for the body. However, during tumorigenesis immune tolerance can represent a barrier for appropriate induction of an efficient immune response aimed at eliminating transformed cells and preventing tumor progression and malignancy. Therefore, knowledge on understanding the mechanisms to enhance or reduce immune tolerance for the design of therapies have been garnered much attention. Tolerance-inducing therapies can be applied to treat auto-immune diseases and allergies as well as transplant rejection, while reduction of tumor-associated tolerance can render tumors more permissive to immune attack.

Sialic acids as therapeutic target to increase tumor immunogenicity

Many tumors tend to express aberrant glycans that are crucially implicated in different steps of tumorigenesis, including tumor cell proliferation, invasion, metastasis and angiogenesis[1]. In particular, tumors present high levels of sialic acids (Sias), which especially contribute to tumor cell dissociation from the primary tumor, tumor dissemination into the bloodstream and formation of metastasis at distant sites by interacting with selectins expressed on platelets and endothelial cells[2]. Given these aspects, Sias represent an attractive target for the treatment of malignancies as therapies directed to reduce tumor sialylation, contributing then to restrain tumor progression and metastasis.

Sias are terminal sugars that have been described to negatively affect the activation of the innate immune cells[2]. Given the poor immunogenicity of sialylated tumors, in **chapter 2** we examined whether there is a direct relation between tumor hyper-sialylation and impaired anti-tumor immune response. We showed that reducing levels of α2-6-linked Sias on murine melanoma B16-OVA by knocking down *SLC35A1* gene via a shRNA approach ($B16^{SLC35A1}$) did not

affect *in vitro* tumor behaviour such as migration and proliferation compared to high sialylated B16-OVA that was transfected with a non-targeting shRNA ($B16^{scrambled}$). Yet, the reduction of Sias on tumors notably delayed tumor growth *in vivo*, indicating that the slower development of $B16^{SLC35A1}$ tumors was influenced by the host's environment. Upon the examination of the tumor microenvironment, we observed that the reduction of sialylation on B16-OVA correlated with lower frequencies of $CD4^+$ $FoxP3^+$ T cells. This effect was only observed at the tumor site. Analysis of tumor draining lymph nodes (TDLNs) did not reveal a difference in the frequency of $CD4^+$ $FoxP3^+$ T cells between $B16^{SLC35A1}$ and $B16^{scrambled}$ tumors. This could suggest that tumor hyper-sialylation might lead to the expansion of naturally occurred Tregs (nTregs), which are recruited to the tumor sites through tumor- produced chemokines such as CCL5, CCL17 and CCL22[3]. Here the expansion of Tregs can occur after the interaction of Sias on tumors with Siglecs expressed on nTregs. However, the expression of Siglecs on nTregs remains unclear.

DCs and other components of the innate immunity such as NK cells and macrophages are crucial players in the recognition as well as elimination of transformed cells in the first phase of cancer immunoediting[4] (**chapter 2**). Tumors such as melanoma and colon carcinoma have been associated with tolerogenic DCs (tDCs), which are characterized by anti-inflammatory phenotype and functions due to low expression of co-stimulatory molecules. These intra-tumoral tDCs promote Treg induction through the secretion of IL-10, TGF-β[5,6] and Indoleamine 2,3-dioxgenase (IDO) as well as by binding PD-1 receptor on T cells via PDL-1 receptor[5,6]. Upon analysis of the intra-tumoral immune composition 18 days after tumor transplantation in mice, no difference in numbers of DCs between $B16^{SLC35A1}$ and $B16^{scrambled}$ was found. Although we did not analyze the phenotype of these intra-tumoral DCs, culturing DCs with $B16^{SLC35A1}$ in vitro did not alter the expression level of co-stimulatory molecules such as CD80, CD40 or CD70 compared to DCs cultured with $B16^{scrambled}$. In contrast, we observed higher amounts of the pro-inflammatory cytokine IL-6 in DC-$B16^{SLC35A1}$ culture, suggesting that DCs in contact with $B16^{SLC35A1}$ are more pro-inflammatory than DCs cultured with the. $B16^{scrambled}$. Moreover, the analysis of cytokines present in the supernatant of DC- $B16^{SLC35A1}$ cultures did not reveal

higher levels of the anti-inflammatory cytokines IL-10 or TGF-β. Surprisingly our data described in **chapter 3 and 4** demonstrate also a direct immune inhibitory effect of Sias on DCs, which leads to a modulation of DC functions. In particular, we showed that Sia-modified antigens taken up by DCs induced a tolerogenic programming which was associated with a de novo generation of $CD4^+$ and $CD8^+$ Tregs at the expense of effector T cell generation. Under these conditions, no altered cytokine levels were observed. In **chapter 3**, we also found that DCs taking up Sia-modified antigens are not able to suppress the functions of activated T cells in a transwell experiment, suggesting that these DCs exert their tolerogenic functions by a cell-contact-dependent mechanisms rather than secretion of immunosuppressive soluble factors. This could explain why DCs that were cultured with $B16^{SLC35A1}$ did not present higher levels of the anti-inflammatory cytokine IL-10 and TGF-β than DC-$B16^{scrambled}$-co-culture, suggesting that hyper-sialylated tumors are characterized by tolerogenic tDCs that can promote Treg induction in a cell-contact manner. All together, these data may indicate that the reduction of Sias on B16-OVA renders DCs pro-inflammatory rather than tolerogenic correlating with the consequent increased anti-tumor T cell responses. During the analysis of tumor-infiltrating immune composition 18 days post tumor implantation in mice, we were surprised to observe higher numbers of NK cells and an increase of IFN-γ at the tumor site of $B16^{SLC35A1}$ tumors. NK cells play a crucial role in the innate recognition and elimination of forming tumors through the direct killing and the secretion of the effector cytokine IFN-γ[7,8]. It has been shown that the treatment of fibrosarcoma with sialidases, which remove terminal Sia residues from glycan chains, enhanced IFN-γ secretion and the killing properties of NK cells[9]. In **chapter 2**, we showed that in vitro cultures of splenocytes of naive mice with $B16^{SLC35A1}$ increased the IFN-γ production by NK cells. Furthermore, increased amounts of IFN-γ was also observed when isolated NK cells from spleens were cultured in vitro with $B16^{SLC35A1}$(**chapter 2**). These data indicate that the tumor milieu of $B16^{SLC35A1}$ tumors, which show reduced sialic acid expression, is enriched for IFN-γ-producing NK cells. It has been shown that depletion of Tregs in tumor-bearing mice led to tumor rejection that was dependent on NK cells, and IFN-γ, which increased the numbers of tumor-infiltrating T cells, indicating that intra-tumoral

Tregs have immunosuppressive on NK cells[10]. Thus reduced numbers of Tregs within $B16^{SLC35A1}$ may lead to less impairment of NK cell functions.

Murine NK cells have been reported to express sialic acid binding receptors such as Siglecs that negatively modulate NK function via putative ITIMs present in their cytoplasmic tail[11]. More specifically, murine NK cells express Siglec-E and Siglec-G, which bind α2-6-linked Sia moieties[11]. Thus, the reduction of α2-6-linked Sias on B16-OVA melanoma may likely minimize the inhibition of the NK effector function, which normally occurs through the interaction of tumor expressed Sias with Siglec expressing NK cells. In addition, given its immunostimulatory properties[12], IFN-γ secretion by NK cells can stimulate and attract new NK cells at $B16^{SLC35A1}$ tumor site, leading to increased accumulation of intra-tumoral NK cells. NK cell-produced IFN-γ also contributes to the DC-mediated $CD4^+$ and $CD8^+$ T cell activation[13-15]. In **chapter 2**, depletion of NK cells in $B16^{SLC35A1}$-bearing mice reduced the numbers of intra-tumoral effector $CD4^+$ and $CD8^+$ T cells, indicating that IFN-γ secreted by NK cells can stimulate DCs to activate T cell response. Indeed, DCs within $B16^{SLC35A1}$ presented an immunogenic rather than tolerogenic phenotype as detected by higher production of pro-inflammatory cytokines IL-6 and TNF (**chapter 2**). Therefore, the reduced numbers of tumor-associated Tregs within $B16^{SLC35A1}$ tumors may also derive from the increased inflammatory status within the tumor determined by the high frequency of NK cells and high levels of IFN-γ. Culturing $B16^{SLC35A1}$with different concentrations of IFN-γ in vitro increased the levels of IP-10 in the supernatant, which has been reported to attract effector T cells in tumor site (**chapter 2**)[16]. This suggests that IFN-γ may also have an effect on the B16 tumor itself, illustrating a direct effect of Sias on the function of NK cells affecting the expression of chemokines to attract effector T cells. The multidirectional effects of tumor hyper-sialylation on NK cells as well as the induction of tumor-associated Tregs in tumor bearing mice, creating a tolerogenic tumor microenvironment is depicted in Figure 1.

In conclusion, we provide evidence that the hyper-sialylation of tumors directly correlates with a tolerogenic tumor microenvironment rendering tumors poorly immunogenic through the modulation of DC function, NK cell activation and Treg induction. Therefore, strategies addressed to reduce levels of Sias on tumors

can potentiate anti-tumor immunity to hinder and restrain tumor growth. Reducing tumor sialylation can also be considered as combinational therapy when combined for instance with chemotherapy and radiotherapy in order to increase anti-tumor immunity and delay of tumor progression. A few glycan-based strategies addressed to reduce tumor hyper-sialylation have been tested in clinical trials. One of these clinical trials comprised the use of Swainsonine, which is a potent inhibitor of the Golgi-mannosidase leading to the inhibition of the N-Glycans synthesis in the Golgi[1,17]. Since Sias are mainly the terminal sugars of N-glycan chains, the use of Swainsonine reduces sialylated N-glycans on tumor surfaces with consequent reduction of tumor growth and invasion[3]. Despite good effects

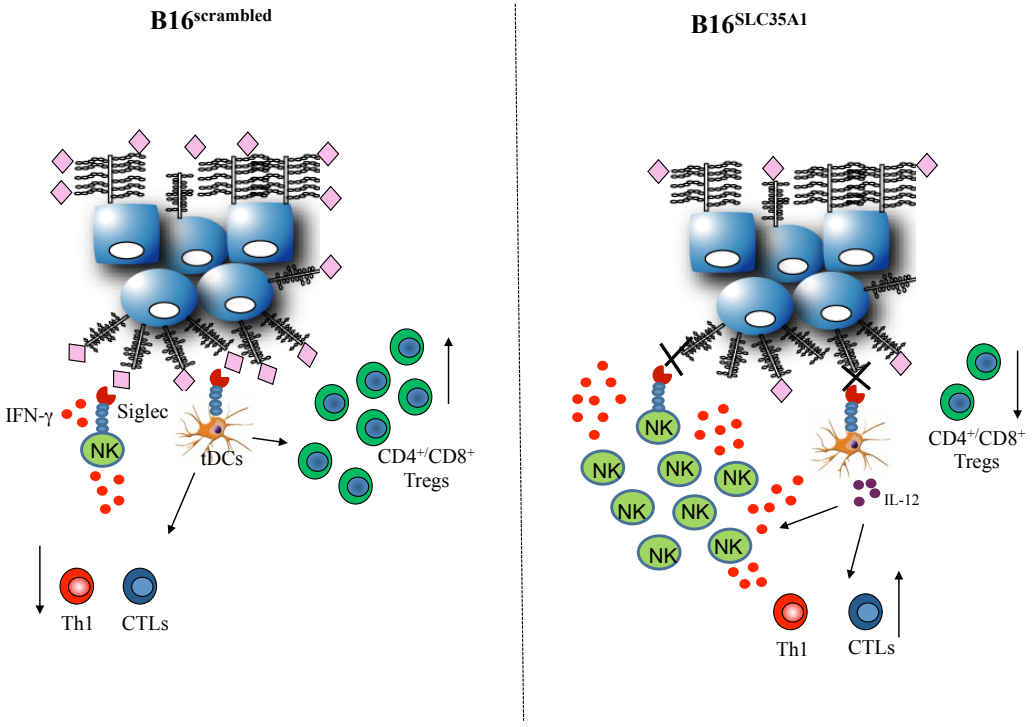


Figure 1: The consequences of sialylation on anti-tumor immunity and the induction of innate and adaptive tumor immunity. The reduction of Sia levels on B16-OVA by knocking down SLC35A1 gene favourably effects tumor immunogenicity by reducing tumor-infiltrating $CD4^+$ and $CD8^+$Tregs and increasing the innate function of intra-tumoral NK cells, as well as local levels of IFN-γ. Tumor-recognizing NK cells secrete IFN-γ, which recruits more NK cells to the tumor site, as well as induce the maturation of DCs, which polarize T cells towards effector $CD4^+$ and $CD8^+$ T cell subsets. The decreased levels of Sias on B16-OVA may also minimize the interactionof Sias with Siglecs expressed on DCs, leading to less tolerogenic DCs and consequent reduction of the DC-mediated Treg induction at the tumor site.

on reducing tumor progression in a phase I clinical trial with patients suffering metastatic carcinoma or leukaemia, the disadvantage of this Swainsonine-based treatment is its non-specific and systemic effect on the biosynthesis of N-glycans in the body. This can lead to severe liver and pancreatic dysfunction in treated patients.

Sialic acids as therapy to resolve immunopathology

Despite their deleterious effects in limiting anti-tumor immunity and herewith contributing to tumor progression, Tregs have an essential and pivotal role in the maintenance of immune tolerance in order to avoid unwarranted and excessive immune response that would be harmful for the body. The beneficial functions of Tregs in immune homeostasis have been especially supported by studies demonstrating that selective deletion of FoxP3$^+$ Tregs in mice was responsible of severe multi-organ autoimmunity, and death within 10 days, while the transfer of Tregs in athymic mice prevented autoimmunity[18,19]. In **chapter 3**, we showed that CD4$^+$ FoxP3$^+$ Tregs can be generated in vitro and in vivo by DCs internalizing Ovalbumin modified with Sias ($DC_{Sia\text{-}OVA}$). In addition, the uptake of Sia- OVA antigen by DCs significantly prevented differentiation of naive CD4$^+$ T cells into IFN-γ-producing effector CD4$^+$ T cells compared to DCs receiving unmodified Ovalbumin (DC_{OVA}), diverting thus CD4$^+$ T cell polarization towards Tregs. Here, we also showed that $DC_{Sia\text{-}OVA}$ could also differentiate Tregs from naive CD4$^+$ T cells of BALB/c DO11.10 mice, demonstrating that the tolerogenic effect of Sia-OVA on T cell differentiation was even visible in in the Th2-prone BALB/c mice and not strain-dependent. Moreover, $DC_{Sia\text{-}OVA}$ were able to promote Treg differentiation from naive CD4$^+$ T cells of BALB/c DO11.10 x Rag$^{-/-}$ tg mice, which lack of natural Tregs (nTregs), suggesting a relevant role of Sia-OVA in the de novo generation of Tregs.

Our studies also revealed a crucial role of the Sia-binding receptor Siglec-E in mediating the tolerogenic effects by the uptake of Sia-OVA in the DC-mediated T cell activation (**chapter 3**). In fact, the absence of this receptor on DCs significantly impaired the binding and uptake of Sia-OVA by DCs as well as potently arrested the generation of CD4$^+$ FoxP3$^+$ T cells in vitro (chapter

3). Nevertheless, we observed that using Siglec-E$^{-/-}$ DCs uptake of Sia-OVA yielded an effect similar to native OVA resulting in T cells skewed towards IFN-γ-producing effector cells. These data indicate that both the induction of Tregs and the inhibition of IFN-γ effector CD4^{+} T cells by Sia-OVA are dependent on its interaction with Siglec-E.

In **chapter 3**, we also demonstrated that the administration of Sia-OVA in mice prior to sensitization with OVA, mixed with poly (I:C) and anti-CD40 increased numbers of Tregs. Similar results were obtained via adoptive transfer of $DC_{Sia\text{-}OVA}$ in mice that were boosted with OVA and CPG, indicating that the promotion of tolerance was independent by the type of adjuvant used, which suggests the potential of inducing tolerance in the presence of different DC stimuli and polarizing signals. Notably, the injection of Sia-OVA in mice not only promoted tolerance, but, as we observed ex vivo, it potently reduced the generation of IFN-γ producing effector CD4^{+} and CD8^{+} T cells. Currently, it is not clear whether the dampening of effector CD4^{+} and CD8^{+} T cell response observed in Sia-OVA treated mice resulted from the immunosuppressive actions of the increased Treg population found or derived from endogenous DCs that became tolerogenic following Sia-OVA injection.

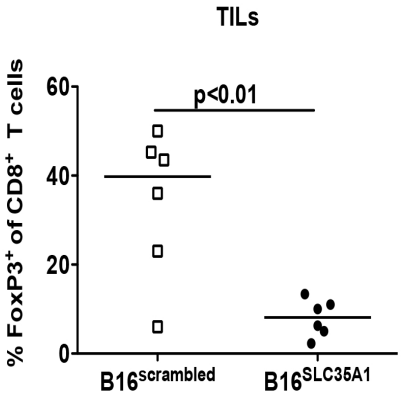
Several protocols based on the manipulation of Tregs have been developed to resolve or reduce immunopathology such as rheumathoid arthritis (RA), systemic lupus erythematosus (SLE) and type 1 diabetes[20-22]. Tregs from type 1 diabetes patients were isolated and expanded in vitro with anti-CD3 and anti-CD28 microbeads and IL-2, and then transferred into patients. Although these expanded Tregs were capable of suppressing T cell functions *in vitro*, they were characterized by loss of FoxP3 expression. These expanded Tregs required the addition of rapamycin to maintain lineage purity and FoxP3 expression[22]. Tregs have also been de novo generated in vitro under the effect of anti-inflammatory mediators such as TGF-β, IL-10, and rapamycin[23]. However, these in vitro generated Tregs were also characterized by the instability of Foxp3 expression, which limited the adoptively transfer of these cells into the patients[24]. According to the tolerogenic effects of Sia-OVA *in vivo*, the treatment of immune-related disease patients with Sia-modified antigens could have the advantage to endogenously induce tolerance and inhibit deleterious CD4^{+} and CD8^{+} effector T

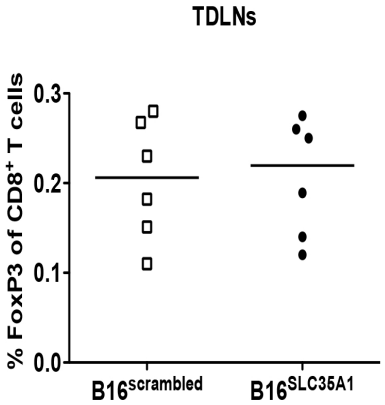
cells.

Although Tregs have been prevalently considered a $CD4^+$ T cell subset, the concept of regulatory T cells has been also expanded to $CD8^+$ T cells[25,26]. Despite initial difficulties in phenotypical and marker characterization, the important role of $CD8^+$ Tregs in immune homeostasis has become evident in knockout mice, where lacking of $CD8^+$ T cells led to auto- immune diseases such as EAE and RA[27-29]. In **chapter 4**, we further investigated the effects of Sia-modified antigen in the induction of $CD8^+$ Tregs by DCs. We found that $DC_{Sia\text{-}OVA}$ led to $CD8^+$ $FoxP3^+$ Treg generation, which was not observed with DC_{OVA} stimulation, while potently inhibited the proliferation of $CD8^+$ T cells and suppressed their differentiation in effector cytotoxic T lymphocytes as revealed by low amounts of intracellular Granzyme B (GrB) and IFN-γ. The observation that Sia-OVA uptake by DCs promoted $CD8^+$ $FoxP3^+$ Treg induction correlates with our findings from $B16^{SLC35A1}$ microenvironment, which was characterized by lower frequencies of $CD8^+$ $FoxP3^+$ T cells 18 days after tumor transplantation besides the reduced number of $CD4^+$ $FoxP3^+$ T cells (box 1). However, as for $CD4^+$ $FoxP3^+$ T cells, numbers of $CD8^+$ $FoxP3^+$ T cells found in the TDLNs of $B16^{SLC35A1}$ and $B16^{scrambled}$ were similar (box 1). Thus, the reduction of hyper-sialylation in B16-OVA also affected the $CD8^+$ T cell subsets with tolerogenic features. Therefore, the generation of $CD4^+$ and $CD8^+$ Tregs by $DC_{Sia\text{-}OVA}$ detected in **chapter 3** and **chapter 4** further suggests that the hypersialylation of tumor has also a direct tolerogenic effect on intra-tumoral DCs leading to the DC-mediated Treg induction. Besides exerting their immunesuppressive function by secretion of IL-10 and TGF-β, $CD8^+$ Treg by cell contact-mechanism dependent mediated by CTLA-4, CD39[30,31]. The over- expression of ectonucleotidase CD39 we observed on $CD8^+$ T cells that were primed by $DC_{Sia\text{-}OVA}$ may suggest that Sia-OVA on DCs induces a $CD8^+$ $FoxP3^+$ Treg population that can exert their immunosuppressive functions through the degradation of the extracellular ATP. However, The specific functionalities of these $CD8^+$ $FoxP3^+$ T cells still need to be elucidated. Although we demonstrated the beneficial effects of sialylated antigen in a prophylactic setting in vivo in **chapter 3**, future studies are needed to assess whether Sia-modified antigen can prevent or reduce the severity of auto-immune disorders in animal models. Preliminary data ex vivo show that the modification

BOX 1. Reduction of Sias on B16-OVA remarkably reduces the frequence of intra-tumoral $CD8^+$ $FoxP3^+$ T cells.

Since reducing Sias on B16-OVA impaired the numbers of intra-tumoral $CD4^+$ Tregs, we also examined the tumor milieu of $B16^{SLC35A1}$ and $B16^{scrambled}$ for $CD8^+$ $FoxP3^+$ T cells 18 days after tumor transplantation. As for $CD4^+$ $FoxP3^+$ T cells, low levels of Sias on B16-OVA led to a reduced frequencies of $CD8^+$ $FoxP3^+$ T cells in $B16^{SLC35A1}$ microenvironment when analysing the tumor infiltrating lymphocytes (TILs) but not in TDLNs. In conclusion, our data show that reducing hypersialylation of B16 caused a decreased numbers of $CD4^+$ and $CD8^+$ Tregs while leading to accumulation of NK cells and increased levels of IFN-γ.





of EAE-inducing peptide Myelin Oligodendrocyte Glycoprotein (MOG) with Sias reduced the proliferation of splenocytes deriving from mice with severe EAE disease. Moreover, to determine whether the modification of antigens with Sias could have clinical application to treat disorders characterized by detrimental immune reactions such as auto-immune and inflammatory diseases, we started to investigate the effects of Sia-OVA on the fate of $CD4^+$ (**chapter 3**) and $CD8^+$ (**chapter 4**) T cells under inflammatory conditions in vitro. In particular, we showed that the co-addition of Sia-OVA and LPS to DCs did not alter the ability of Sia-OVA to induce $CD4^+$ Treg antigen and prevent effector $CD4^+$ T cell differentiation (**chapter 3**). By contrast, the addition of LPS to DCs and Sia-OVA clearly inhibited the generation of $CD8^+$ $FoxP3^+$ T cell population, whereas the induction of effector $CD8^+$ T cells was still impaired (**chapter 4**). However, the simultaneous administration of Sia-OVA and LPS to DCs did not lead to different expression levels of MHC-I and MHC-II compared to homeostatic conditions (**chapter 3**). This can exclude a lower antigen-presentation in the context of MHC-I, which could explain the enduring $CD8^+$ Treg induction under inflammatory conditions. In **chapter 3 and 4**, we only assessed the contribution of LPS on Sia-OVA functions on $CD4^+$ and $CD8^+$ T cell differentiation However, the contribute and the effects of other TLR triggering such as TLR3, and TLR 7 needs to be investigated to better elucidate the effects of sialylation under inflammatory conditions.

In **chapter 3**, we demonstrate that DCs promoted $CD4^+$ Treg generation and prevented effector $CD4^+$ T cell response when Sias covalently coupled to OVA, while the incubation of DCs with a mixture of Sias and OVA did not produce the same effect as Sia-OVA antigen. Moreover, the Treg induction depended on the presence of Sias on OVA, since coupling OVA with an other glycan such as GlcNac promoted the differentiation of naive OT-II $CD4^+$ T cells into Th1 but not into Tregs (**chapter 3**). The importance of the OVA conjugation with Sias on DC-mediated Treg induction suggests that effects of the Sia coupling to the antigen could derive from a different intracellular routing of the antigen or an alteration in the antigen processing. Nevertheless, the equal expression levels of MHC-I and MHC-II we observed between DC_{OVA} and $DC_{Sia\text{-}OVA}$ may exclude that $CD4^+$ and $CD8^+$ Treg induction by $DC_{Sia\text{-}OVA}$ was a result of lower antigen presentation.

The intracellular routing of the sialylated antigen on DCs needs to be investigated to better elucidate and unravel the tolerogenic effects of the sialylated antigen.
In this thesis, we also started to characterize $DC_{Sia\text{-}OVA}$ in order to unveil the mechanisms through which Sia-OVA drives DCs to divert $CD4^+$ and $CD8^+$ T cell differentiation towards $CD4^+$ Tregs and $CD8^+$ Tregs, respectively. Different processes leading to tDCs have been extensively described[32]. Although immature tissue-resident DCs promote tolerance in the absence of danger[33], DCs can become tolerogenic if matured in the presence of anti-inflammatory mediators such as IL-10, TGF-β and tend to secrete anti-inflammatory factors that polarize naive T cells towards Tregs. However, tDCs can also promote Treg generation via engagement of inhibitory molecules such as Immunoglobulin-like transcript (ILT)-3 and - 4 and Programmed cell death ligand (PD-L)1 and 2[34,35]. Analysis of supernatants from DCs receiving Sia-OVA did not reveal increased amounts of IL-10 and TGF-β, but the uptake of Sia-OVA by DCs drastically reduced the secretion of pro-inflammatory cytokines such as IL- 6, and TNF-α. The observation that $DC_{Sia\text{-}OVA}$ can negatively affect DC_{OVA}-mediated IFN-γ^+ $CD4^+$ T cell generation only if they are in contact with naive $CD4^+$ T cells (**chapter 3** and **5**) supports the hypothesis that a ligand expressed on DCSia-OVA is involved in the modulation of T cell activation. So far, the analysis of the surface co-stimulatory and tolerogenic molecules did not reveal a different signature of $DC_{Sia\text{-}OVA}$ compared to DCOVA. Despite these results, the gene expression profile of $DC_{Sia\text{-}OVA}$ in chapter 5 revealed upregulation of the tDC-associated ILT-3 gene. ILT-3 is a member of Immunoglobulin superfamily inhibitory ILTs. ILT-3 protein is mainly expressed on myeloid antigen presenting cells, and, similarly to Siglecs, it contains ITIMs, which transduce inhibitory signals[36]. ILT3 is upregulated on DCs in response to immunosuppressive signalling such as IL-10, vitamin D and immunosuppressive $CD8^+$ T cells. ILT3-overexpressing DCs show a reduced expression of co-stimulatory molecules and are responsible of anergic T cells[37]. It has been described that tolerogenic DCs in transplant recipients express high levels of ILT-3 and ILT-4, which inhibit the suppressor functions of alloreactive $CD4^+$ and $CD8^+$ T cells inducing Tregs and T cell anergy[37,38]. Therefore, although we did not observe downregulation of CD86 and other co-stimulatory molecules, it is possible that $DC_{Sia\text{-}OVA}$ induce tolerance via ILT-3.

The analysis of gene expression profile of $DC_{Sia\text{-}OVA}$ confirmed the involvement of Siglec-E in the tolerogenic effects of Sia-OVA were also confirmed through the gene expression profile of $DC_{Sia\text{-}OVA}$. In particular, based on the gene array, DCs receiving Sia-OVA showed an expression of Siglec-E 102 fold higher than DCs taking up unmodified OVA (**chapter 5**). Interaction of Siglecs with Sias induces tyrosine phosporylation of ITIM domain by Src- family kinases followed by the recruitment of SHIP-1/2 and SHP-1/2, which subsequently leads to the inhibition of immunoreceptor tyrosine-based activation motifs (ITAMs)-dependent signaling. Although Siglec-E was highly expressed in $DC_{Sia\text{-}OVA}$, genes involved in downstream signaling of Siglec-E were not differentially expressed in $DC_{Sia\text{-}OVA}$. Siglecs are receptor involved in cell signalling as well as antigen uptake for antigen presentation and processing[39]. Therefore, the induced up-regulation of Siglecs may further occur in order to increase the uptake of Sia-antigens. Additionally, the interaction of Sia-OVA with Siglec-E can then induce inhibitory signalings into $DC_{Sia\text{-}OVA}$ leading to ILT-3-overexpressing tDCs with a reduced secretion of pro-inflammatory cytokines and promoting Treg induction. However, further characterization of $DC_{Sia\text{-}OVA}$ as well as their mechanisms used to modulate T cell responses await future investigations. The possible roles of Sia-OVA in DC-mediated T cell differentiation are illustrated in Figure 2.

Concluding remarks and future directions

The data presented in this thesis provide new insights in the role of Sias in the modulation of the immune response. More specifically, in this thesis we show that Sias on antigen divert the DC-mediated T cell differentiation towards $CD4^+$ and $CD8^+$ Tregs preventing the generation of effector $CD4^+$ and $CD8^+$ T cell response *in vitro* and *in vivo*. In addition, here we show that the high Sia levels in tumor directly correlate with a Treg-rich tumor microenvironment that renders tumors poorly immunogenic and less permissive to the immune attack as reducing tumor hyper-sialylation results in decreased frequency of intra-tumoral $CD4^+$ and $CD8^+$ Tregs. The reduced tumor hyper-sialylation also leads to an increased accumulation of tumor- infiltrating NK cells in the early stage of tumor development. Besides, the increased IFN-γ found within Sia^{low} tumors can suggest the presence of IFN-γ-producing NK cells that induce maturation of DCs leading to the generation

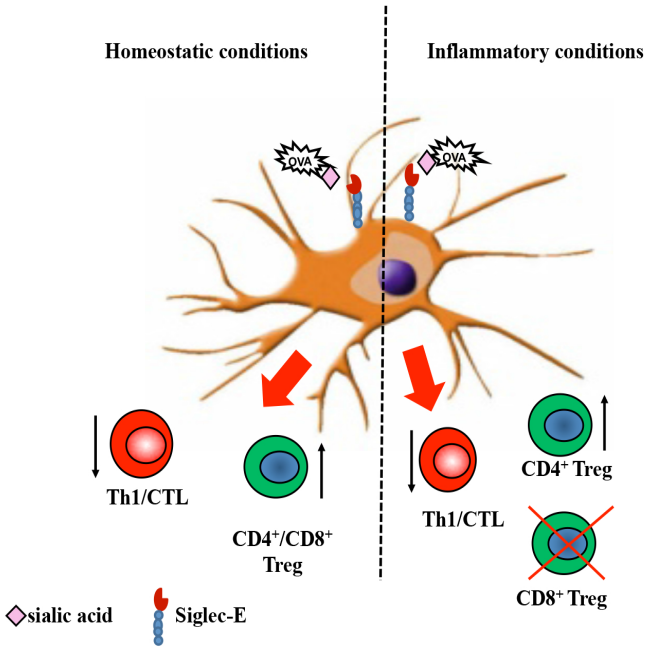


Figure 2: The effects of sialic acid modified antigens in Treg induction and prevention of effector immunity. DCs that take up Sia-modified OVA alter the DC-mediated differentiation of $CD4^+$ and $CD8^+$ T cells towards Tregs, thereby preventing the generation of the effector T cell response. Similar results were obtained even under inflammatory conditions in vitro. However, the generation of $CD8^+$ Tregs was not induced under inflammatory condition.

of effector T cells rather than Tregs.

So far, many studies have been mainly focused on the effects of Sias in the innate immune response[40]. Our thesis introduces new knowledge in the modulatory effects of Sias in T cell activation and differentiation that can lead to future directions for the design of therapies based on the modulation of T cell response to treat diseases. In particular, Sias can have dual therapeutical implications: their targeting on hyper-sialylated tumors can potentiate anti-tumor immune response, while using them to modify disease-associated antigens can be exploited to therapeutically treat auto-immune and inflammatory disorders such as MS, RA and Type 1 Diabetes.

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